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Incorporation of chlorhexidine and nano-sized sodium trimetaphosphate into a glass-ionomer cement: Effect on mechanical and microbiological properties and inhibition of enamel demineralization

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ABSTRACT

Objective: To evaluate the antimicrobial/antibiofilm and mechanical properties, and the effect on enamel demineralization of a resin-modified GIC (RMGIC) containing CHX and nano-sized sodium trimetaphosphate (TMP).

Methods: RMGIC was associated with CHX (1.25 or 2.5%) and/or TMP (7 or 14%). Antimicrobial and antibiofilm activity were assessed using agar diffusion test and evaluation of biofilm metabolism, respectively. In addition, fluoride (F) and TMP releases as well as the diametral tensile (DTS) and compressive (CS) strength were determined. The percentage of mineral loss (%SH), integrated loss of subsurface hardness (ΔKHN) and enamel F concentrations were also evaluated.

Results: RMGICs containing CHX associated or not with TMP presented higher inhibition zones and effect on *S. mutans* biofilm. A reduction on CS was observed only for RMGIC + 2.5%CHX and on DTS for RMGIC + 2.5%CHX + 14%TMP. The highest F and TMP releases and lowest %SH and ΔKHN values were detected for RMGIC + 1.25%CHX + 14%TMP and RMGIC + 2.5%CHX + 14%TMP. Higher enamel F concentrations were observed for TMP groups.

Conclusion: 1.25%CHX and 14%TMP increased antimicrobial/antibiofilm action and the ability to prevent enamel demineralization, with minimal effect on the mechanical properties of RMGIC.

Clinical significance: RMGIC containing CHX and TMP is an alternative material for patients at high risk for dental caries and can be indicated for low-stress regions or provisional restorations.

1. Introduction

In recent years, Dentistry has focused on the search for techniques and materials that allow the absence or minimum removal of enamel and/or carious dentin, in order to conserve the dental remnant and prevent pulpal damage. Thus, different restorative alternatives such as the infiltration of caries lesions in enamel or even partial removal of caries in dentin have arisen [1]. However, this cariogenic residue could contribute to inadequate adhesion of the restorative material to the cavosurface angle and result in the formation of marginal *gaps*. In addition, these *gaps* may also arise due to polymerization contraction, a limitation of the adhesive materials. This space between material and restoration leads to greater retention of dental biofilm or facilitates the passage of nutrients to the remaining bacteria of the carious process, allowing their survival and, consequently, the formation of secondary caries at the margins. In this way, it is necessary to study dental materials with antimicrobial properties, which act on the remaining microbiota and on the entry of new microorganisms into the tooth-restoration interface, and with anticariogenic properties, which promote remineralization and prevent the development of secondary caries.

Nowadays, materials with anticariogenic action are those that present and release fluoride, calcium, or phosphate into the buccal

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medium. The most popular material is glass-ionomer cement (GIC), which has been indicated in the prevention of demineralization, promotion of remineralization of enamel and dentin, and inhibition of the growth of bacteria that cause dental caries [2]. These qualities have been attributed to the release of fluoride, which is deposited in the dental tissues around the restoration, making them more resistant to acid attack. Although there are differences in the fluoride release pattern between conventional GIC and resin-modified GIC (RMGIC), a common feature is the fact that larger fluoride release occurs in the first 24 h, reduces rapidly in the subsequent two weeks, and stabilizes after 5–6 weeks [3]. A continuous release of fluoride could promote higher effect for caries reduction than only an initial burst of fluoride [1]. in situ studies have demonstrated that some fluoride-containing restorative materials, including composites and glass ionomer cements, showed no caries protective effect on surrounding enamel [1,4]. However, hydroxyl-, calcium- and fluoride-containing materials interferes in the demineralization process next the restoration [1].

The association of polyphosphates with the reduction in dental caries began in the 60′s, when it was added to diets, chewing gum and, later, dentifrices [5–7]. Polyphosphates have a high affinity for hydroxyapatite (HA); it prevents the release of ions and reduces the surface area available for dissolution [8,9]. In this way, these polyphosphates produce an anticaries effect on the enamel [7,10]. Sodium trimetaphosphate (TMP) is a cyclic polyphosphate which, when adsorbed on the enamel surface, reduces enamel, enhances enamel remineralization [7,10], decreases hydroxyapatite solubility and mineral exchange [8,9] and changes the affinity between the enamel surface and salivary proteins [11]. A decrease in enamel demineralization has been observed when TMP was added to fluoride products [7,10,12]. In addition, the effect of the addition of TMP to composite resins on demineralization-remineralization processes has been evaluated [13,14]. The combination of 14.1% TMP and fluoride in composite resins resulted in less demineralization; this result was related to increased fluoride release from the materials due to the presence of TMP, with little change in resin hardness [13].

Besides the anti-caries action, the addition of an antimicrobial component to the GIC, such as chlorhexidine (CHX), has been proposed to increase restoration longevity, which would help to reduce/eliminate the remaining microbiota and control the biofilm adjacent to the restoration. In an in vitro/in vivo study, incorporated 1.25% CHX to the GIC without causing a detrimental effect on the mechanical properties or odontoblastic cells [15]. Moreover, this combination completely eliminated *mutans* streptococci after three months of partial caries removal in children. However, other studies have shown that some forms and concentrations of CHX may alter the mechanical properties of GIC [16,17]. One way to diminish the impact of the incorporation of antimicrobial agents on the mechanical properties of GIC would be to reduce particle sizes. To date, there are no studies associating TMP nanoparticles and CHX with GIC.

In order to maintain the anticariogenic property of the GIC, providing ions that participate in the demineralization-remineralization process and promote mineral saturation of the enamel/dentin in the long term, besides to increase antimicrobial properties of GIC, the incorporation of inorganic polyphosphates and CHX could be an interesting strategy. The aim of this study was to verify if the incorporation of nano-sized TMP and CHX into a RMGIC indicated for dental restoration would increase antimicrobial and antibiofilm action and the ability to prevent enamel demineralization, without interfering in the mechanical properties. The null hypothesis of the study was that the incorporation of nano-sized TMP and CHX into a RMGIC would cause no changes in the antimicrobial/antibiofilm effect, physico-mechanical properties, fluoride release, or enamel demineralization.

2. Materials and methods

2.1. Synthesis and characterization of TMP nanoparticles

Nanoparticles of sodium trimetaphosphate (TMP) were prepared at the Interdisciplinary Laboratory of Electrochemistry and Ceramics (Department of Chemistry, Federal University of São Carlos, São Carlos, SP, Brazil) according to Danelon et al. [10]. First, seventy grams of pure (micrometric) TMP ($Na_3O_9P_3$, Aldrich, purity ≥ 95% CAS 7785−84-4) were ball milled using 500 g of zirconia spheres (diameter of 2 mm) in 1 L of isopropanol (Merck, Darmstadt, Deutschland, Germany). After 48 h, the resulting powder was separated from the alcoholic media and ground in a mortar. X-ray diffraction (XRD), using a RigakuDmax 2500 PC diffractometer in the 2θ range from 10 to 80° with a scanning rate of 2°/min, was used to characterize the powder crystallinity and estimate crystallite size (coherent crystalline domains) of the TMP and milled for 48 h.

2.2. Preparation of the modified RMGIC

The RMGIC chosen for the current study was Fuji II LC (GC Corporation, Tokyo, Japan). Chlorhexidine diacetate (Sigma, St. Louis, MO, USA) (CHX), commercially available as a solid substance, was used. In addition, 7 and 14% TMP and 1.25 and 2.5% CHX were incorporated into the RMGIC powder. These concentrations were defined according to Tiveron et al. [13] and Türkün et al. [18], respectively. For the addition of TMP and/or CHX to the RMGIC powder, 100 g of the final product of the mixture (RMGIC + CHX, RMGIC + TMP and RMGIC + CHX + TMP) were considered. The mixture was homogenized using a ball mill (Pulverisette 7, Fritsch, Idar-Obserstein, Germany) at 250 rpm for 5 min using an agate-grinding bowl of 45 mL containing 180 agate balls (5 mm of diameter). In total, eight new composites were obtained the following groups: RMGIC + 1.25%CHX; RMGIC + 2.5%CHX; RMGIC + 7%TMP; RMGIC + 1.25%CHX + 7% TMP; RMGIC + 2.5%CHX + 7%TMP; RMGIC + 14%TMP; RMGIC + 1.25%CHX + 14%TMP, and RMGIC + 2.5%CHX + 14%TMP. Fuji II LC was used as a control (RMGIC). For all experiments, the RMGIC specimens were prepared according to the manufacturer's instructions (3.2 g powder to 1.0 g of liquid) and light activated for 30 s using a halogen-curing unit (Blue Star III, Microdont, Brazil).

2.3. Microbiological tests

2.3.1. Agar diffusion test

Assays were carried out using the following species: *Streptococcus mutans* (ATCC 25175), *Lactobacillus acidophilus* (IAL#523), *Actinomyces israelii* (ATCC 12102), and *Candida albicans* (ATCC 6093). Strains were subcultured on Brain Heart Infusion Agar (BHI; Difco, Le Point de Claix, France) and incubated at 37 °C for 48 h under anaerobic conditions for *S. mutans, L. acidophilus,* and *A. israelii* and under aerobic conditions for *C. albicans*. Subsequently, 5 colonies of each strain were inserted into BHI broth individually 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. The diffusion test on agar was conducted according to Duque et al. [19]. A base layer containing 15 mL of BHI agar mixed with 300 µL of each inoculum was prepared in sterilized Petri dishes (20 mm x 100 mm). After solidification of the culture medium, ten wells (4 mm of diameter) were made in each plate and, in sequence, filled with one of the experimental composites or control material (RMGIC). The materials were prepared (3.2:1.0 ratio), inserted into the wells using a syringe (Centrix, Shelton, USA), and light activated for 30 s. As a control of the experiment, 10 µL of aqueous 0.2% chlorhexidine

digluconate was applied on sterile filter paper discs (n = 6), also 4 mm in diameter. The plates were kept for 2 h at room temperature to allow diffusion of the materials and incubated at 37 °C for 24 h. After this period, two measures of each inhibition zone around the materials were carried out using a digital caliper. Tests were performed in duplicate.

2.3.2. Biofilm assay

Biofilm assay was based in the study of Hu et al. [20], with some modifications. *S. mutans* (ATCC 25175) was cultured anaerobically in BHI broth overnight at 37 °C. The bacterial suspension obtained was adjusted to an optical density of 0.3 at 600 nm (approximately 3×10^8 CFU/mL) with a spectrophotometer (Microplate Spectrophotometer EONC, Biotek, USA), for further usage. For each tested material, fifteen disc-shaped specimens (3 × 2 × 1 mm) were prepared using metal matrices. The specimens were kept for 1 h in an environment with 100% humidity, washed with sterile distilled water, and disinfected under ultraviolet light for 15 min. The specimens were inserted into the wells of a 24-well plate (Corning, NY, USA) and kept in suspension by an orthodontic wire that remained fixed with utility wax on the polystyrene plate cover during the experimental period. A solution of 1 mL BHI supplemented with 1% sucrose and 5 µL bacterial suspension was inoculated into each well. The plates were incubated at 37 °C in an anaerobic chamber for 24 h. Metabolic cell activity of biofilm formed in the wells was quantified by the XTT (2,3 (2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide) (Sigma-Aldrich) reduction assay [21]. For this, the wells containing the formed-biofilm were washed gently with saline solution, and 1 mL of a solution comprising 150 mg/L of XTT and 10 mg/L of phenazine methosulphate (Sigma-Aldrich) was added. The plates were incubated in the dark at 37 °C for 3 h and then 200 µL of the solubilized suspension were transferred to 96-well microplates and the absorbance read at 490 nm. The results were expressed in percentages of biofilm growth, considering a control group without the presence of RMGIC as 100% of biofilm growth. All assays were performed in triplicate on three different occasions.

2.4. Measurements of mechanical tests

2.4.1. Diametral tensile (DTS) and compressive (CS) strength

For the diametral tensile strength (DTS) test, ten specimens were prepared by means of metallic matrices (diameter: 4 mm, height: 6 mm), according to ISO specifications (ISO 9917 − 1:2007) [22]. Samples were kept in an incubator for 1 day and 7 days (d), at 37 °C, with a relative humidity of 100%. After the storage periods, the specimens were submitted to the DTS test. Diametral compression was performed using an Instron universal testing machine (DL3000, Instron, Canton, MA, USA), at a speed of 1 mm/min, in a horizontal position, with a load of 500 N, until fracture occurred. DTS was calculated from the following formula and expressed in MPa: $DTS = 2Fr/\pi Dt$, where Fr was the applied load (N), D the diameter of the samples (mm), and 't' the thickness of the samples (mm).

For the compressive strength (CS) test, ten specimens were prepared using metallic matrices, 2 mm diameter × 4 mm height, according to ISO standards (ISO 9917 − 2:2010) [22]. After the storage periods (24 h and 7 d), the samples were submitted to the CS test in an Instron universal testing machine (Instron), at a speed of 1 mm/min, in the vertical position, with a load of 500 N, until fracture occurred. The CS of the samples was calculated using the following equation: $CS = 2P/\pi dh$ where CS (MPa) was the compressive strength, P (N) the load at fracture, 'd' the diameter of the specimen (mm), and 'h' the thickness (mm).

2.5. Demineralization tests

2.5.1. Preparation and selection of enamel blocks and RMGIC specimens

Enamel blocks (4 × 4 × 3 mm) were obtained from bovine incisors kept in formaldehyde 2%, pH 7.0, for 30 days prior to the experimental procedures. The enamel surfaces were polished using 600, 800, and 1,200-grade water-cooled silicon carbide paper disks (Buehler), with a final polish using a felt disk (Buhler Polishing Cloth 40–7618) moistened with a 1-µm diamond polishing suspension (Extec, Enfield, CT, USA), resulting in the removal of the enamel to a depth of approximately 120 µm. After polishing, blocks were sectioned to 1 mm from the block border (4 × 3 × 3 mm). Blocks were selected according to the initial surface hardness test (SH_1; 320–380 KHN) and randomly divided into ten experimental groups (n = 10, each): Placebo (without RMGIC); RMGIC without CHX/TMP (RMGIC); RMGIC + 1.25%CHX; RMGIC + 2.5%CHX; RMGIC + 7%TMP; RMGIC + 1.25%CHX + 7%TMP; RMGIC + 2.5%CHX + 7%TMP; RMGIC + 14%TMP; RMGIC + 1.25%CHX + 14%TMP; and RMGIC + 2.5%CHX + 14%TMP. Ten specimens (3 mm x 2 mm x 1 mm) were fabricated for each group according to the manufacturer's instructions and photo activated at the top and bottom surfaces for 30 s each.

2.5.2. pH-cycling test

The specimens of each material were attached to the sectioned borders of 90 enamel blocks (n = 10 blocks *per* material) with sticky wax [13]. The blocks/specimens were individually subjected to a pH-cycling regimen of five cycles at 37 °C, being immersed in demineralization (DES) solution (**6 h - 2.2 mL/mm^2; 2.0 mmol/L Ca and P, in acetate buffer 75 mmol/L, 0.04 ppm F, pH 4.7) and remineralization (RE) solution (18 h – 1.1 mL/mm^2; 1.5 mmol/L Ca, 0.9 mmol/L P, 150 mmol/L KCl in cacodylate buffer 20 mmol/L, 0.05 ppm F, pH 7.0)** for 5 days. The blocks were washed with jets of deionized water for 30 s whenever blocks were removed from the pH-cycling solutions. After 5 days, the RE solution was renewed, and the blocks remained in this solution for 48 h [13].

2.5.3. Enamel surface and cross-sectional hardness analysis

Surface hardness was determined with a Shimadzu microhardness tester (HMV-2000, Shimadzu, Kyoto, Japan) with a Knoop diamond indenter under a 25-g load for 10 s [10]. Five equidistant indentations were made, spaced 100 µm from each other to 300 µm from the sectioned enamel border [13]. After the five pH cycles, the materials fixed to the blocks were removed and subjected to the final enamel surface hardness test (SH_2) by producing five other indentations (100 µm from the baseline indentations). These data were used to calculate the percentage of loss of surface hardness ($\%SH = [[SH_2\text{-}SH_1]/SH_1]x100$). For the cross-sectional hardness, blocks were sectioned at the center and one of the halves was included in acrylic resin and gradually polished until the enamel was totally exposed. Afterwards, one sequence of 13 indentations was created at different distances (5, 10, 15, 20, 25, 30, 40, 50, 70, 90, 110, 220, 330 µm) from the surface of the enamel, in the central region of the blocks, using a Micromet 5114 hardness tester (Buehler, Lake Bluff, IL, USA) with a Knoop diamond indenter under a 5-g load for 10 s and the integrated loss of subsurface hardness (ΔKHN) was calculated [13].

2.5.4. Release of fluoride (F) and TMP from materials

Six samples of each material were placed in polypropylene test tubes with lids. The specimens remained suspended using stainless steel wires, inside the tubes. Each tube contained 2 mL of the demineralizing (DES) or remineralizing (RE) solution [13]. Initially, the specimens were stored in DES solution (**2.0 mmol/L Ca and P, in acetate buffer 75 mmol/L, pH 4.7**) for 6 h. **Next, the samples were transferred to new tubes containing RE solution (Ca 1.5 mmol/L, P 0.9 mmol/L, KCl 150 mmol/L in buffer 0.02 mol/L, pH 7.0) for 18 h.**The test tubes were subjected to constant shaking (shaking table TE-420 Orbital–Tecnal, Piracicaba, SP, Brazil) at 37 °C. These procedures were repeated for 15 days. The specimens were washed with deionized water and dried with absorbent paper before being immersed in a new solution. The solutions were collected daily, identified, and stored in polypropylene test tubes at 4 °C to measure F and TMP release.

2.5.5. Analysis of F in the enamel and F and TMP in the DES and RE solutions

The remaining halves of the blocks were sectioned again to obtain 2 × 1 × 3 mm blocks [13] and subjected to a microabrasion procedure using 400-grit silicon carbide paper (Buehler) and acid hydrolysis, as described by Delbem et al. [24]. After removal of a ~50-μm layer of each enamel block, 0.5 mL of HCl 1.0 mol/L was added to the flasks, which were kept under constant shaking for 1 h in an orbital shaker (TE-420; Tecnal Equipamentos, Piracicaba, SP, Brazil) [23]. For F analysis, a specific electrode 9409BN (Thermo Scientific, Beverly, MA, USA) and reference microelectrode (Analyser, São Paulo, Brazil) coupled to an ion analyzer (Orion 720A$^+$, Thermo Scientific, Beverly, MA, USA) were used. The electrodes were previously calibrated with standard solutions (0.25 a 4.00 μg F/mL), under the same conditions as the samples. The reading was performed using 0.25 mL of the biopsy solution, buffered with the same volume of TISAB II modified NaOH 1.0 mol/L [25]. Results were expressed as μg F/mm^3.

For the F analysis in the DES and RE solutions, a specific electrode 9409BN and a reference microelectrode coupled to an ion analyzer were also used. However, the electrodes were calibrated with standards containing from 0.5–64 μg F/mL. Thus, an aliquot of 0.5 mL of the DES and RE solutions was buffered with the same volume of TISAB II. Results were expressed as μg F/cm^2.

TMP released from materials in the DES and RE solutions was determined after acid hydrolysis. For this, aliquots of 0.1 mL of the solutions were added to 0.1 mL of hydrochloric acid (HCl) 1.0 mol/L and heated in water at 100 °C for 1 h. After hydrolysis, phosphorus (P) was determined by the molybdate method [25], using aliquots of 20 μL of the solutions, added to a mixture of 50 μL molybdate and 20 μL reactive reducer. P concentration in the DES and RE solutions, after acid hydrolysis, was subtracted from the quantity of P present in the DES (57.5 ± 7.6 μg P/mL) and RE solutions (26.9 μg P/mL) and the final value was considered as being the TMP present in the solutions. Results were converted to μg TMP/cm^2. For all days (15 days), F and TMP concentrations of the DES and RE solutions were determined separately and, subsequently, added (DES + RE), completing a period of 24 h [13].

2.6. Statistical analysis

For the mechanical tests (diametral tensile and compressive strength) and F and TMP release in the solutions (DES + RE), materials and time were considered as variation factors. All data, with the exception of inhibition zones, biofilm assay, %SH and ΔKHN, presented normal (Kolmogorov–Smirnov) and homogeneous (Cochran) distribution and were submitted to two-way analysis of variance followed by the Bonferroni correction or Student-Newman-Keuls' test. For the microbiological tests, the Kruskal-Wallis and Mann-Whitney tests were applied. The data from %SH and KHN were subjected to Kruskal-Wallis followed by Student-Newman-Keuls' test. Analyses were performed using SigmaPlot 12.0 with a significance level of 5%.

3. Results

3.1. Antibacterial activity

The mean values (standard deviation) of the inhibition zones for each tested material, according to the evaluated strains, are shown in Table 1. In relation to the control group (RMGIC), there was a significant increase in the inhibitory action of RMGIC when incorporated with CHX, regardless of concentration (1.25 or 2.5%), for all tested microorganisms ($p < 0.05$). The materials with CHX and 7 or 14% TMP did not differ statistically from the GICs without TMP. On the other hand, the cements containing only TMP (7 or 14%) were similar to the RMGIC (control group).

Fig. 1 shows the results of XTT reduction for single biofilms of *S. mutans* formed on the wells in the presence of the evaluated RMGICs. When CHX (1.25 and 2.5%) or CHX and TMP (7 and 14%) were added to the RMGIC, there was a reduction in the metabolic activity of biofilm cells, compared with the control group (RMGIC) ($p < 0.05$). It can be observed that the groups RMGIC + 1.25%CHX, RMGIC + 2.5%CHX, RMGIC + 1.25%CHX + 7%TMP, and RMGIC + 2.5%CHX + 7%TMP were similar to each other and presented better results than the RMGIC ($p < 0.05$). In short, the group RMGIC + 2.5%CHX + 14%TMP group led to better results in comparison with the other groups ($p < 0.05$).

3.2. Measurements of mechanical tests

The means (standard deviation) of the values obtained for mechanical testing in the different periods (1 day and 7 days) are shown in Table 2 . In relation to the compressive strength (CS) test, after 1 day, TMP (in both concentrations) incorporated into the cements with or without CHX promoted a reduction in the values of CS, with RMGIC + 2.5%CHX + 14%TMP group presented the lowest values ($p < 0.05$). After 7 days, there was an increase in the values of CS for all groups, except by RMGIC + 2.5%CHX. It was noted that the RMGIC, RMGIC + 1.25%CHX. RMGIC + 7%TMP and RMGIC + 1.25%CHX + 7%TMP groups were similar to each other. For the diametral tensile strength (DTS) test, after 1 day, the RMGIC groups did not differ from each other and from the control ($p > 0.05$). After 7 days, there was a slight increase in the values of DTS for the control RMGIC group. A reduction on DTS was observed only for RMGIC + 2.5%CHX + 14% TMP.

3.3. Demineralization tests

3.3.1. Release of fluoride (F) and TMP from materials

In relation to the release of F, the highest values were observed on the first day for all tested groups ($p < 0.05$) (Fig. 2). The average of the released total fluoride in the experimental period (15 days) was higher for the RMGIC + 1.25%CHX + 14%TMP group followed by the RMGIC + 14%TMP group ($p < 0.05$). From the fourth day, all groups presented constant release. The total quantity of released phosphorus (TMP) is shown in Fig. 3. Higher values were observed on the first day for all groups. There was a gradual increase in the release of TMP in the groups with 7% TMP, regardless of CHX concentration ($p < 0.05$). The RMGIC + 14%TMP and RMGIC + 2.5%CHX + 14%TMP groups presented greater TMP release.

3.3.2. Surface and cross-sectional hardness and F in the enamel

Table 3 shows the results of the percentage of surface hardness loss (%SH), integrated loss of subsurface hardness (ΔKHN), and F in the enamel. The RMGIC + 14%TMP, RMGIC + 1.25%CHX + 14%TMP, and RMGIC + 2.5%CHX + 14%TMP groups presented lower mineral loss (%SH) in relation to the other groups ($p < 0.05$). The materials with 14% TMP associated with CHX (in both concentrations) presented lower values of ΔKHN when compared to the other groups ($p < 0.05$). The RMGIC + 7%TMP and RMGIC + 14%TMP groups presented higher F concentration in the enamel in relation to the other groups ($p < 0.05$) and the RMGIC + 1.25%CHX and RMGIC + 2.5%CHX + 7%TMP groups were similar to the RMGIC.

4. Discussion

In the current study, the addition of CHX (1.25 and 2.5%) increased the inhibitory effect of RMGIC against microorganisms related to caries development, such as S. *mutans*, L. *acidophilus*, *A. israelii,* and *C. albicans*. Moreover, the addition of TMP (7 or 14%) did not interfere with the antimicrobial activity of CHX. However, these combinations decreased physico-mechanical properties of RMGIC. Based on these results, the null hypothesis was partially rejected. CHX diacetate was chosen due to its powdery appearance and the fact that it can be easily

Table 1
Mean values (SD) of the diameter of the inhibition zone (mm, $n = 10$) for the different microorganisms according to the groups.

Groups	*S. mutans*	*L. acidophilus*	*A. israelii*	*C. albicans*
RMGIC	6.18 ± 1.1^{A}	5.22 ± 0.4^{A}	5.51 ± 0.6^{A}	5.80 ± 0.6^{A}
RMGIC + 1.25%CHX	12.71 ± 1.2^{B}	9.60 ± 0.6^{B}	13.86 ± 1.2^{B}	9.91 ± 1.4^{B}
RMGIC + 2.5%CHX	12.72 ± 1.1^{B}	10.25 ± 0.5^{B}	13.89 ± 0.8^{B}	11.50 ± 1.5^{B}
RMGIC + 7%TMP	6.65 ± 1.4^{A}	4.94 ± 0.4^{A}	5.58 ± 0.6^{A}	6.54 ± 1.4^{A}
RMGIC + 1.25%CHX + 7%TMP	12.45 ± 0.6^{B}	10.61 ± 0.8^{B}	13.97 ± 0.7^{B}	10.38 ± 0.7^{B}
RMGIC + 2.5%CHX + 7%TMP	12.50 ± 0.7^{B}	11.05 ± 0.5^{B}	15.28 ± 0.9^{B}	9.34 ± 0.7^{B}
RMGIC + 14%TMP	7.54 ± 2.2^{A}	4.94 ± 0.4^{A}	5.86 ± 1.0^{A}	6.20 ± 0.9^{A}
RMGIC + 1.25%CHX + 14%TMP	13.44 ± 0.9^{B}	10.76 ± 0.5^{B}	14.62 ± 0.7^{B}	8.66 ± 0.6^{B}
RMGIC + 2.5%CHX + 14%TMP	13.27 ± 1.3^{B}	10.52 ± 0.6^{B}	14.28 ± 0.5^{B}	8.9 ± 0.6^{B}
CHX**	21.87 ± 1.7^{C}	17.48 ± 0.7^{C}	24.58 ± 2.1^{C}	25.76 ± 3.2^{C}

**CHX - 0.2% chlorhexidine digluconate.
* Different letters indicate statistical difference between the groups of materials, according to ANOVA and Bonferroni tests ($p < 0.05$).

blended with GIC; in addition to this, it is a more stable antibacterial material and not prone to decomposition [27]. Studies have demonstrated excellent antibacterial, physical, and adhesive properties of RMGIC containing CHX diacetate [18,27]. The addition of CHX diacetate into a RMGIC resulted in an increase in the long-term antibacterial properties over the conventional ionomer cement alone for *S. mutans and* L. *acidophilus*, but not for *C. albicans* [18]. In parallel, this study also demonstrated that the addition of TMP (7 or 14%) did not interfere with the antimicrobial activity of CHX. In our study, agar plate diffusion was the method of choice, as is relatively inexpensive and can be performed rapidly and easily with many specimens. However, this method does not simulate the clinical condition where multiple species of bacteria will be growing in complex biofilms [18]. Considering this, we also evaluated the effects of these new materials on the metabolic activity and cellular viability of *S. mutans* biofilms formed on the wells and specimens, respectively. RMGIC containing CHX, in both concentrations, reduced significantly the metabolic activity of *S. mutans* biofilms. It was observed that, RMGIC + 2.5%CHX + 14%TMP demonstrated the best results, probably due to higher release of CHX from RMGIC more structurally modified by incorporation of the highest concentrations of CHX and TMP. Mechanical tests confirmed that this combination affected severally compressive strength, diametral tensile strength and hardness of original GIC. Considering each agent separately, this study also demonstrated that the association of chlorhexidine at 1.25% did not cause damage to the mechanical properties tested, at 1 day and 7 days. The same was not observed for the concentration of 2.5% chlorhexidine, which mainly affected the compressive strength. When TMP and CHX were combined, a reduction in the diametral tensile strength and compression strength was noted. Thus, although the best combination for antimicrobial and antibiofilm effects was RMGIC + CHX 2.5% + TMP 14%, this combination presented the worst performance in relation to mechanical properties. The use of a lower concentration of this antimicrobial agent is also interesting for the cytotoxic potential of chlorhexidine, and is known to influence the mechanical properties of cement at higher concentrations [15,17,18]. The inclusion of different components in the glass ionomer cement could interfere with the reaction of polyacrylic acid and glass particles. In addition, small changes in the powder-liquid ratio could also affect the physico-mechanical properties of the material [15].

Previous study has demonstrated that hydroxyl-, calcium- and fluoride-containing restorative materials have more effect on demineralization next to restorations than fluoride-containing materials [1]. Recent investigations also showed an increased ability to reduce mineral loss and enamel remineralization when inorganic phosphate,

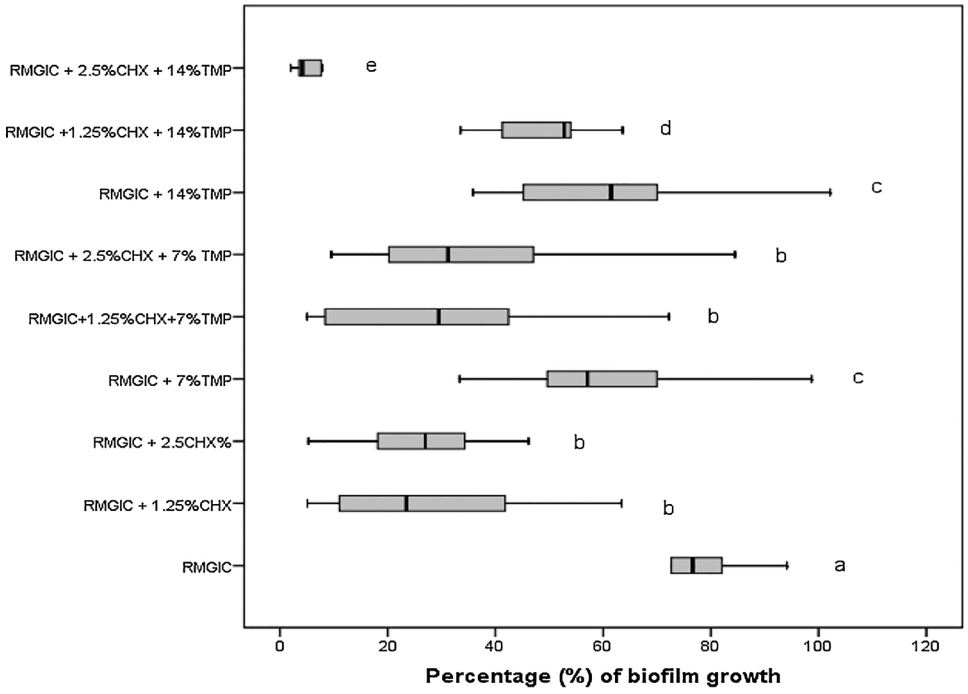

Fig. 1. Percentage (%) of biofilm growth of *S. mutans* formed on the wells in the presence of the evaluated RMGIC.

Table 2
Mean values (SD) of the compressive and diametral tensile strength according to the groups (n = 10).

Groups	Compressive strength (MPa)		Diametral tensile (MPa)	
	1-day	7-days	1-day	7-days
RMGIC	116.9 ± 13.8^{A,a}	188.3 ± 21.5^{A,b}	68.0 ± 22.7^{A,a}	84.6 ± 8.9^{A,b}
RMGIC + 1.25%CHX	114.1 ± 15.6^{A,a}	186.5 ± 9.0^{A,b}	68.45 ± 7.7^{A,a}	62.5 ± 16.9^{A,a}
RMGIC + 2.5%CHX	108.0 ± 24.7^{A,a}	107.7 ± 25.6^{B,a}	68.5 ± 15.3^{A,a}	58.0 ± 10.7^{A,a}
RMGIC + 7%TMP	96.0 ± 4.3^{A,a}	168.9 ± 25.4^{AC,b}	60.6 ± 11.3^{A,a}	60.1 ± 6.0^{A,a}
RMGIC + 1.25%CHX +7%TMP	92.9 ± 6.6^{B,a}	151.3 ± 26.1^{AC,b}	60.710.7^{A,a}	60.5 ± 11.8^{A,a}
RMGIC + 2.5%CHX +7%TMP	88.5 ± 7.5^{B,a}	130.9 ± 34.0^{BC,b}	52.8 ± 6.8^{A,a}	56.5 ± 10.2^{A,a}
RMGIC + 14%TMP	94.2 ± 12.6^{B,a}	137.7 ± 25.5^{BC,b}	59.3 ± 9.2^{A,a}	65.3 ± 7.2^{A,a}
RMGIC + 1.25%CHX +14%TMP	94.6 ± 12.6^{B,a}	111.8 ± 16.6^{B,b}	56.8 ± 10.3^{A,a}	49.4 ± 6.8^{A,a}
RMGIC + 2.5%CHX +14%TMP	71.7 ± 16.2^{C,a}	134.0 ± 19.7^{BC,b}	70.7 ± 11.1^{A,a}	51.6 ± 8.9^{A,b}

[A] Different upper letters indicate statistical difference among the groups, according to ANOVA and Bonferroni test ($p < 0.05$).
[a] Different lower letters indicate statistical difference between the periods, according to t Student test ($p < 0.05$).

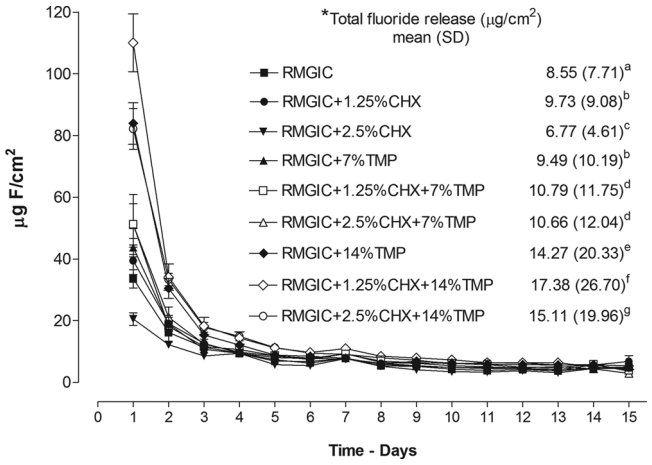

Fig. 2. Total released Fluoride (F) (ug/cm^2) by RMGIC in the DES and RE solutions after 15 days. Vertical bars show the standard deviation of the means.

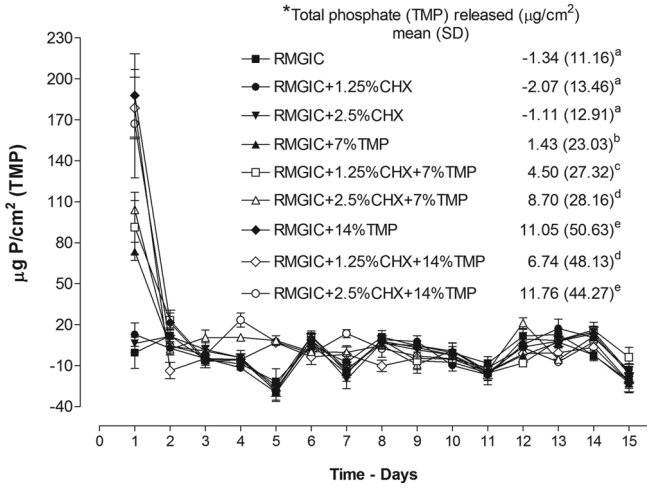

Fig. 3. Total released Phosphorus (TMP) (ug/cm^2) by RMGIC in the DES and RE solutions after 15 days. Vertical bars show the standard deviation of the means.

sodium trimetaphosphate (TMP), was associated in different fluoride formulations [7,12,28]. A similar synergistic effect between TMP and fluoride in reducing demineralization and induction of remineralization was observed when they were associated in composite resin [13,14], although concentrations above 14% of TMP affected the hardness of the material. In the present study, the incorporation of 14% TMP had a beneficial effect on the reduction of the enamel demineralization both at the surface and in the subsurface, regardless of the concentration of CHX, showing that the antimicrobial agent did not affect this property. Other phosphates are also being inserted into glass ionomer cement, such as casein phosphorous-amorphous calcium phosphate (CPP-ACP) and an increase in enamel remineralization has been shown in the area adjacent to the restoration after pH cycling, although a reduction in compressive strength and diametral traction, and prolongation of cement healing time were also observed [29].

Table 3
Mean values (SD) of the percentage surface hardness loss (%SH), integrated loss of subsurface hardness (ΔKHN) and fluoride (F) from enamel according to the groups (n = 12).

Groups	Analysis		
	%SH (KHN)	ΔKHN (KHN x µm)	F (µg/mm^3)
Placebo	−87.9^{a}	6,620.6^{a} (935.1)	0.78^{e}
RMGIC	−73.6^{b,c}	3,029.7^{c} (423.1)	2.48^{a}
RMGIC + 1.25%CHX	−74.0^{b}	2,014.1^{d} (251.4)	2.06^{a,b}
RMGIC + 2.5%CHX	−72.1^{b,c}	4,986.3^{b} (685.4)	1.37^{c}
RMGIC + 7%TMP	−74.2^{b,c}	1,932.2^{d} (529.1)	3.46^{d}
RMGIC + 1.25%CHX + 7%TMP	−71.7^{c}	2,223.5^{d} (741.4)	1.17^{c}
RMGIC + 2.5%CHX + 7%TMP	−75.1^{b}	2,968.3^{c} (439.9)	1.88^{a,b}
RMGIC + 14%TMP	−69.6^{d}	1,712.1^{d} (519.1)	2.98^{d}
RMGIC + 1.25%CHX + 14%TMP	−68.4^{d}	1,296.3^{d} (174.7)	1.61^{b,c}
RMGIC + 2.5%CHX + 14%TMP	−69.8^{d}	1,578.1^{d} (379.3)	1.45^{b,c}

[a] Different letters indicate statistical difference between the groups according to the analysis (%SH, ΔKHN and F: Bonferroni or Student-Newman-Keuls tests).

In this study, it was decided to incorporate two agents simultaneously in the glass ionomer cement, chlorhexidine and TMP, with the future clinical goal of suppressing residual infection and increasing the longevity of the restoration in minimally invasive approaches. It was verified that the presence of TMP did not interfere in the antimicrobial activity of chlorhexidine and the same was observed for chlorhexidine in relation to the reduction in enamel demineralization, both surface and subsurface, by TMP, for which the best results were observed with a concentration of 14%. The simultaneous inclusion of these agents in the cement had no negative effect on the mechanical properties of the material when compared to the cements containing only TMP in the corresponding concentrations 7 or 14%, except for the combination 2.5% CHX and 14% TMP for the mechanical tests. This combination was more detrimental to the mechanical properties of the material,

possibly due to the larger incorporation of TMP and CHX in the powder of the material and the greater change in the powder composition of the ionomer cement. This also possibly influenced the best antibiofilm action of this mixture, since it allowed larger release of components present in the GIC, probably due to the increase in the solubility of the material. Thus, the inclusion of both TMP and CHX agents in suitable concentrations could have a beneficial effect on the GIC.

In this present study, there was a significant F increase with the inclusion of TMP, demonstrating the same effect observed when inserted in other vehicles, such as dentifrices, varnishes, gels, and even composite resin [7,12–14,28]. The glass ionomer cement group combined with 1.25% CHX and 14% TMP showed the highest fluoride release, practically double that observed in the control group. Glass ionomer cements have the same pattern of high release at the first 24 h, rapidly decreasing on day 2 and reaching gradually decreasing levels in the first two weeks [13–30]. This higher availability of fluoride in the medium presented an effect on the reduction of enamel demineralization, since the groups that presented the best anticaries effects were those that presented greater fluoride release. The same was observed for the release of phosphorus (TMP) from the materials, which was increased from the isolated 7% TMP incorporation, except for the group RMGIC + 1.25% CHX + 14% TMP which demonstrated similar phosphorus release to the group with 2.5% CHX and 7% TMP. This variation did not affect the ability to prevent demineralization of this group, which presented similar values to the other two groups with 14% TMP. Studies have shown that TMP and fluoride can act independently and simultaneously in the process of enamel demineralization [7,12]. In this study, in order to maintain the powder-liquid ratio of the material, the amount of cement powder corresponding to that inserted from the chlorhexidine salts and/or TMP was removed and it is possible components of the powder were reduced, including fluoride. However, there was no influence on the ability to reduce demineralization by comparing the groups containing 14% TMP alone or with chlorhexidine at the two concentrations. TMP, due to its cyclic conformation, remains adhered to the enamel for a longer period than linear polyphosphates. This could explain the inhibitory effect of TMP on demineralization, even in isolation or in the presence of low concentrations of fluoride. The presence of CHX at 2.5% clearly affected ΔKHN, but only in absence of TMP. Chlorhexidine gluconate, CHX acetate and fluoride are absorbed on to in vitro hydroxyapatite and enamel power. The affinity of CHX gluconate for hydroxyapatite is higher than that of CHX acetate. The structure of CHX molecule is similar to that of an amino acid and the existence of negatively charged centers in the molecule increase the affinity for calcium sites. Besides, the amount of fluoride adsorbed to hydroxyapatite is reduced in the presence of CHX, possibly because the competitive adsorption of F and CHX on the same binding sites on the hydroxyapatite [31]. Due to synergic effect between TMP and F, high concentrations of TMP could increase the affinity of F for hydroxyapatite and reduce enamel demineralization.

Our results corroborate with those obtained by Cheng et al. [32], respecting the fact that the anticaries agents themselves (CaF_2 and ACP) and the methodology used are different from the one used in the present study, including other mechanical tests (modulus of elasticity and flexural strength) and other microbiological analyzes. In the present study, the use of nanoparticles of TMP was chosen, with the intention of reducing the interference of the incorporation of this phosphate in the physical-mechanical and microbiological properties of the GIC, as occurred in the study developed with another inorganic phosphate, hexametaphosphate, also on a nanometer scale associated with CHX, which maintained the antimicrobial action of both agents without significantly altering the diametral tensile strength of the RMGIC [16].

5. Conclusions

It is concluded that the glass ionomer cement associated with 1.25% CHX and 14% TMP presented the most promising results, showing a positive effect on the reduction of surface and enamel subsurface demineralization, an increase in antimicrobial activity and effect against biofilm of *S. mutans*, the release of fluoride from the ionomer cement, and with minimal reduction in the mechanical properties and fluoride release of the dental enamel compared to the original RMGIC.

Conflict of interest

The authors have no conflict of interest.

Acknowledgments

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References

[1] A.M. Kielbassa, J. Muller, C.R. Gernhardt, Closing the gap between oral hygiene and minimally invasive dentistry: a review on the resin infiltration technique of incipient (proximal) enamel lesions, Quintessence Int. 40 (2009) 663–681.
[2] T.M. Hafshejani, A. Zamanian, J.R. Venugopal, Z. Rezvani, F. Sefat, M.R. Saeb, H. Vahabi, P. Zarrintaj, M. Mozafari, Antibacterial glass-ionomer cement restorative materials: a critical review on the current status of extended release formulations, J. Control. Release 262 (2017) 317–328, https://doi.org/10.1016/j.jconrel.2017.07.041.
[3] H.K. Yip, W.T. Lam, R.J. Smales, Fluoride release, weight loss and erosive wear of modern aesthetic restoratives, Br. Dent. J. 187 (1999) 265–270.
[4] G.E. Dijkman, J. de Vries, J. Arends, Secondary caries in dentine around composites: a wavelength-independent microradiographical study Caries Res, Caries Res. 28 (1994) 87–93.
[5] R.S. Harris, A.E. Nizel, N.B. Walsh, The effect of phosphate structure on dental caries development in rats, J. Dent. Res. 46 (1967) 290–294, https://doi.org/10.1177/00220345670460012901.
[6] G.L. Vogel, Z. Zhang, C.M. Carey, A. Ly, L.C. Chow, H.M. Proskin, Composition of plaque and saliva following use of an alpha-tricalcium-phosphate-containing chewing gum and a subsequent sucrose challenge, J. Dent. Res. 79 (2000) 58–62, https://doi.org/10.1177/00220345000790010901.
[7] E.M. Takeshita, M. Danelon, L.P. Castro, K.T. Sassaki, A.C. Delbem. Effectiveness of a Toothpaste with Low Fluoride Content Combined with Trimetaphosphate on Dental Biofilm and Enamel Demineralization in situ, Caries Res. 49 (2015) 394–400, https://doi.org/10.1159/000381960.
[8] A.C. Delbem, J.A. Souza, A.C. Zaze, E.M. Takeshita, K.T. Sassaki, J.C. Moraes, Effect of trimetaphosphate and fluoride association on hydroxyapatite dissolution and precipitation in vitro, Braz. Dent. J. (25) (2014) 479–484, https://doi.org/10.1590/0103-6440201300174.
[9] J.G. Amaral, J.P. Pessan, J.A.S. Souza, J.C.S. Moraes, A.C.B. Delbem, Cyclotriphosphate associated to fluoride increases hydroxyapatite resistance to acid attack, J. Biomed, Mater. Res. B. Appl. Biomater. 106 (2018) 2553–2564, https://doi.org/10.1002/jbm.b.34072.
[10] M. Danelon, J.P. Pessan, F.N. Neto, E.R. de Camargo, A.C. Delbem, Effect of toothpaste with nano-sized trimetaphosphate on dental caries: In situ study, J. Dent. 43 (2015) 806–813, https://doi.org/10.1016/j.jdent.2015.04.010.
[11] H. Nordbö, G. Rölla, Desorption of salivary proteins from hydroxyapatite by phytic acid and glycerophosphate and the plaque-inhibiting effect of the two compounds in vivo, J. Dent. Res. 51 (1972) 800–811, https://doi.org/10.1177/00220345720510031701.
[12] M. Danelon, E.M. Takeshita, L.C. Peixoto, K.T. Sassaki, A.C. Delbem, Effect of fluoride gels supplemented with sodium trimetaphosphate in reducing demineralization, Clin. Oral Investig. 18 (2014) 1119–1127, https://doi.org/10.1007/s00784-013-1102-4.
[13] A.R. Tiveron, A.C. Delbem, G. Gaban, K.T. Sassaki, D. Pedrini, Effect of resin composites with sodium trimetaphosphate with or without fluoride on hardness, ion release and enamel demineralization, Am. J. Dent. 26 (2013) 201–206.
[14] A.R. Tiveron, A.C. Delbem, G. Gaban, K.T. Sassaki, D. Pedrini, In vitro enamel remineralization capacity of composite resins containing sodium trimetaphosphate and fluoride, Clin. Oral Investig. 19 (2015) 1899–1904, https://doi.org/10.1007/s00784-015-1404-9.
[15] A.R. de Castilho, C. Duque, T.C. Negrini, N.T. Sacono, A.B. de Paula, C.A. de Souza Costa, D.M. Spolidório, R.M. Puppin-Rontani, In vitro and in vivo investigation of the biological and mechanical behavior of resin-modified glass-ionomer cement containing chlorhexidine, J. Dent. 41 (2013) 155–163, https://doi.org/10.1016/j.jdent.2012.10.014.
[16] E.R. Hook, O.J. Owen, C.A. Bellis, J.A. Holder, D.J. O'Sullivan, M.E. Barbour, Development of a novel antimicrobial-releasing glass ionomer cement functionalized with chlorhexidine hexametaphosphate nanoparticles, J. Nanobiotechnology 23 (2014) 2–9, https://doi.org/10.1186/1477-3155-12-3.
[17] L.M. Marti, M.D. Mata, B. Ferraz-Santos, E.R. Azevedo, E.M. Giro, A.C. Zuanon, Addition of chlorhexidine gluconate to a glass ionomer cement: a study on

mechanical, physical and antibacterial properties, Braz. Dent. J. (25) (2014) 33–37, https://doi.org/10.1590/0103-6440201302328.
[18] L.S. Türkün, M. Türkün, F. Ertuğrul, M. Ateş, S. Brugger, Long-term antibacterial effects and physical properties of a chlorhexidine-containing glass ionomer cement, J. Esthet. Restor. Dent. 20 (2008) 29–44, https://doi.org/10.1111/j.1708-8240.2008.00146.x.
[19] C. Duque, T.C. Negrini, N.T. Sacono, D.M. Spolidorio, C.A. de Souza Costa, J. Hebling, Clinical and microbiological performance of resin-modified glass-ionomer liners after incomplete dentine caries removal, Clin. Oral Investig. (13) (2009) 465–471, https://doi.org/10.1007/s00784-009-0304-2.
[20] J. Hu, X. Du, C. Huang, D. Fu, X. Ouyang, Y. Wang, Antibacterial and physical properties of EGCG-containing glass ionomer cements, J. Dent. Res. (41) (2013) 927–934, https://doi.org/10.1016/j.jdent.2013.07.014.
[21] S. Silva, M. Henriques, T. Oliveira, D. Williams, J. Azeredo, In vitro biofilm activity of non-*Candida albicans* species, Curr. Microbiol. 61 (2010) 534–540, https://doi.org/10.1007/s00284-010-9649-7.
[22] ISO, Dentistry – Water-based Cements – Part 1: Powder/liquid Acid-base Cements, ISO 9917-1, Geneva, 2007.
[23] A.C. Delbem, M. Danelon, K.T. Sassaki, A.E. Vieira, E.M. Takeshita, F.L. Brighenti, E. Rodrigues, Effect of rinsing with water immediately after neutral gel and foam fluoride topical application on enamel remineralization: an in vitro study, Arch. Oral Biol. 55 (2010) 913–918, https://doi.org/10.1016/j.archoralbio.2010.07.020.
[24] K.M. Alves, J.P. Pessan, F.L. Brighenti, K.S. Franco, F.A. Oliveira, M.A. Buzalaf, K.T. Sassaki, A.C. Delbem, In vitro evaluation of the effectiveness of acidic fluoride dentifrices, Caries Res. (41) (2007) 263–267, https://doi.org/10.1159/000101915.
[25] G. Dalpasquale, A.C.B. Delbem, J.P. Pessan, G.P. Nunes, L.F. Gorup, F.N.S. Neto, E.R. de Camargo, M. Danelon, Effect of the addition of nano-sized sodium hexametaphosphate to fluoride toothpastes on tooth demineralization: an in vitro study, Clin. Oral Investig. (21) (2017) 1821–1827, https://doi.org/10.1007/s00784-017-2093-3.
[26] C.H. Fiske, Y. Subbarow, The colorimetric determination of phosphorus, J. Biol. Chem. 66 (1925) 375–400.
[27] S. Jaidka, R. Somani, D.J. Singh, S. Shafat, Comparative evaluation of compressive strength, diametral tensile strength and shear bond strength of GIC type IX, chlorhexidine-incorporated GIC and triclosan-incorporated GIC: An in vitro study, J. Int. Soc. Prev. Community Dent. (6) (2016) S64–S69, https://doi.org/10.4103/2231-0762.181188.
[28] M.M. Manarelli, A.C. Delbem, T.D. Binhardi, J.P. Pessan, In situ remineralizing effect of fluoride varnishes containing sodium trimetaphosphate, Clin. Oral Investig. (19) (2015) 2141–2146, https://doi.org/10.1007/s00784-015-1492-6.
[29] F. Shafiei, B. Yousefipour, H. Farhadpour, Marginal microleakage of a resin-modified glass-ionomer restoration: Interaction effect of delayed light activation and surface pretreatment, Dent. Res. J. (Isfahan) (12) (2015) 224–230.
[30] M.F. Cabral, R.L. Martinho, M.V. Guedes-Neto, M.A. Rebelo, D.G. Pontes, F. Cohen-Carneiro, Do conventional glass ionomer cements release more fluoride than resin-modified glass ionomer cements? Restor. Dent. Endod. 402 (2015) 209–215.
[31] D. Ben-Yaakov, M. Friedman, Z. Hirschfeld, I. Gedalia, Fluoride enhancement of chlorhexidine uptake by hydroxyapatite and enamel powders, J. Oral Rehabil. 11 (1984) 65–70.
[32] L. Cheng, M.D. Weir, H.H. Xu, A.M. Kraigsley, N.J. Lin, S. Lin-Gibson, X. Zhou, Antibacterial and physical properties of calcium-phosphate and calcium-fluoride nanocomposites with chlorhexidine, Dent. Mater. (28) (2012) 573–583, https://doi.org/10.1016/j.dental.2012.01.006.