



The association of chlorhexidine digluconate and calcium chloride to use as vehicle of a silicate calcium-based cement

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Abstract

Objective: The purpose of this study was to evaluate the influence of the addition of 2% chlorhexidine digluconate (CHX) associated with 5% calcium chloride (CaCl₂) on antimicrobial activity, setting time, pH and calcium release of gray mineral trioxide aggregate (GMTA).

Materials and Methods: GMTA powder was mixed with water, 2% CHX alone or 2% CHX combined with 5% CaCl₂. Antimicrobial activity was determined against *Enterococcus faecalis* (ATCC 29212) strains by agar diffusion test. Data obtained were submitted to kruskal wallis tests. Analysis of the setting time was evaluated by American society for testing and materials C266-03 requirements. The pH and calcium release analysis were evaluated, in 24 h, 7, 14 and 28 days using pH meter equipment and atomic absorption spectrophotometer, respectively. Data obtained were analyzed by ANOVA, in 5% significance level.

Results: Significant differences were seen ($P < 0.01$) among the zones of bacterial growth inhibition produced by 5% CaCl₂ + 2% CHX combination against *E. faecalis* when compared with water ($P < 0.05$). Regarding the setting time, that combination had the shortest setting time ($P < 0.05$). All associations were alkaline and released calcium. No statistical difference was observed between the experimental groups at the different periods of analysis ($P > 0.05$).

Conclusion: Combination of 5% CaCl₂ + 2% CHX reduced the setting time and enhanced the antimicrobial activity of GMTA without changing the pH and calcium release.

Introduction

Mineral trioxide aggregate (MTA) is available as white MTA (WMTA) or gray MTA (GMTA) powder, composed of thin hydrophilic particles whose main components are tricalcium silicate, tricalcium aluminate, tricalcium oxide, and silicate oxide.^[1] There are also small quantities of other mineral oxides like bismuth oxide that provides the physical and chemical properties to the aggregate.^[2]

GMTA has higher concentrations of Al₂O₃ (+ 122%), MgO (+ 130%), and especially FeO (+ 1000%) compared with those of WMTA.^[1] The calcium oxides present in MTA have

the ability to dissociate into calcium and hydroxyl ions, resulting in higher pH and calcium ions release.^[3] This dissociation is important because both biocompatibility and induction of mineralized tissue formation of root canal filling materials depend on such properties.^[4]

The GMTA (Angelus, Londrina, PR, Brazil) available in the Brazilian dental market is composed of 80% Portland cement and 20% bismuth oxide. The calcium sulfate present in the pro root MTA was not added in the angelus composition, thus reducing the setting time.^[5]

The antimicrobial activity of MTA-based cements is lower than sealapex with zinc oxide, zinc oxide and eugenol, and

sealer 26.^[6] Some substances have been recommended to improve the antimicrobial activity of MTA cements, such as 0.12% or 2.0% chlorhexidine digluconate (CHX).^[7,8] Addition of CHX to MTA causes no change in the biological properties of MTA, but can decrease the compressive strength and enhance the setting time.^[8-10]

The combination of CHX and NaOCl has been advocated to enhance their antimicrobial properties.^[11] However, when mixed, NaOCl and CHX produce an orange-brown precipitate that stains the walls of the pulp chamber and contains the parachloroaniline.^[12] A possible alternative would be the addition of calcium chloride (CaCl₂) to the CHX because the presence of chlorine could enhance the antimicrobial effect of CHX, as MTA vehicle.

CaCl₂ at various concentrations have been added to MTA to reduce its setting.^[10] Although MTA has many favorable properties that support its clinical use, it has a long setting time ranging from 75 min to 6 h.^[1,13] The long setting time may favor the solubility and/or disintegration or displacement of MTA from the retrograde cavity.^[5] The addition of 10% CaCl₂ to MTA produces reduction of the initial setting time of cements by 50% and by 35.5%-68.5% in the final setting time.^[14] Despite these considerations, there are no explanations about whether the addition of CHX alone or in association with CaCl₂ interferes with antimicrobial activity and physico-chemical properties of GMTA, such as pH and calcium release.

The purpose of this study was to evaluate the effect of the addition of 2% CHX and 5% CaCl₂ + 2% CHX (1:1, v/v) on antimicrobial activity, setting time, pH and calcium release of GMTA (Angelus Soluções Odontológicas, Londrina, PR, BR).

Materials and Methods

In all experiments, 1 g of GMTA (G) (Ref. 820, Angelus Soluções Odontológicas) was mixed with 0.30 ml of aqueous solutions (vehicle). The vehicles used were: G1-deionized water (control group) (Araraquara Dental School, Araraquara, SP, Brazil), G2- 2% CHX (Arte e Ciência, Araraquara, SP, Brazil), and G3-2% CHX (Arte e Ciência, Araraquara, SP, Brazil) in combination with 5% CaCl₂ (Dinâmica Química Reagentes, Diadema, SP, Brazil) (0.15 ml: 0.15 ml). MTA-cements were manipulated in accordance with the manufacturer's instructions.

Antimicrobial Activity

Strains used as indicators of antimicrobial activity were the following: *E. faecalis* (ATCC 29212) and *Micrococcus luteus* (ATCC 9341). Evaluation was done in quadruplicate by using the agar diffusion test. The well method was conducted on double-layered plates. The base layer was composed of 10.0 ml sterilized müller-hinton agar (MH; Difco, Detroit, MI, USA) poured into 20 × 100 mm sterilized petri plates. After solidification, 5.0 ml of the seed layer, obtained by addition of the inoculums at a concentration of 10⁶ colony-forming u/ml to 5.0 ml of MH, was added. Thereafter, five 4-mm wells (one for each material, one for 2% CHX as positive control and one for saline solution as

negative control) were made by removing the agar at equidistant points and then filled immediately with the materials to be evaluated. The plates (100 mm × 10 mm) were maintained at room temperature for 2 h to allow pre-diffusion of the materials, and then incubated at 37°C for 24 h. Aliquots of 5.0 ml prepared with 1% agar (Difco) and 0.05% triphenyltetrazolium chloride (Sigma, St. Louis, MO, USA) were added for optimization. After solidification, they were incubated at 37°C for 30 min. This technique allows differentiating between areas of microbial growth (red areas) and diffusion zones. The inhibition zones around the wells were then measured with a millimeter ruler with accuracy of 0.5 mm. Data obtained was subjected to Kruskal-Wallis and Dunn tests at a 5% significance level.

Determination of the Setting Time

The setting time of the cements was determined according to American society for testing and materials (ASTM) C266-03 (Philadelphia: ASTM; 2008 specification) and measured under controlled temperature and humidity: 37 ± 1°C and 95 ± 5% relative humidity.^[15] The cements were mixed and inserted into metallic ring molds (10 mm diameter and 2 mm thickness), with three specimens fabricated for each cement. At 180 s, each specimen was indented with a gilmore needle (113.5 g) until the initial setting time was determined. Then, a gilmore needle weighing 456.5 g was used to determine the final setting time. The setting times were determined as being the time elapsed from the beginning of the mixing to the time at which no indentation was detected on the surface of the specimens. Data obtained were subjected to ANOVA test at a significance level of 5%.

Analyses of pH and Calcium Release

Thirty polyethylene tubes measuring 0.5 cm in length and 1.0 mm in internal diameter were filled with the mixtures to be evaluated by using a Lentulo spiral (Maillefer, Ballaigues, Switzerland). For pH and calcium release evaluation, 10 samples were prepared for each group (G) of material being studied.

The tubes filled with fresh mixtures were weighed in order to check the standardization of the amount of cement. They were placed in polypropylene flasks (Injeplast, São Paulo, SP, Brazil) containing 10 ml of deionized water (neutral pH) and kept in oven at 37°C (Farmen, São Paulo, SP, Brazil). Prior to immersion of the specimens, both pH and calcium concentration of deionized water were verified, with pH being 6.8 and calcium being totally absent. Evaluations of pH and calcium ion release were carried out after 24 h, 7, 14 and 28 days. After each period of evaluation, the tubes were removed and placed into another flask with the same volume of new deionized water.

Measurement of pH was performed with a pH meter (model DM22, Digimed, São Paulo, SP, Brazil), previously calibrated with solutions of known pH (4, 7, and 14), at constant temperature (25°C). After the removal of the specimens, the flasks were placed in a shaker (251, Farmen) for 5 s before pH measurement. The control procedure included measuring the pH of the water in which no specimens had been immersed.

Calcium release was measured by using an atomic absorption spectrophotometer (AA6800, Shimadzu, Tokyo, Japan) equipped with a calcium hollow cathode lamp as describe by Vasconcelos *et al.*^[16] Data obtained were subjected to statistical analysis by using ANOVA test at significance level of 5%.

Results

Table 1 shows the antimicrobial activity of the materials tested. The results demonstrated that *E. faecalis* (ATCC 29212) and *M. luteus* (ATCC 9341) were inhibited by all groups. Analysis of *E. faecalis* showed that the combination of 5% CaCl₂ + 2% CHX (G3) with GMTA promoted greater antimicrobial activity zone compared to MTA with water (G1) ($P < 0.05$). No significant difference was observed between 2% CHX alone (G2) or in combination with 5% CaCl₂ (G3) ($P > 0.05$). There was also no difference between 2% CHX (G2) and control group (G1) ($P > 0.05$). The positive control group (2% CHX) presented antimicrobial activity zone of 23.0 mm, whereas the negative control group present no antimicrobial activity zone.

Regarding the setting time [Table 2], G1 had the shortest initial and final setting time ($P < 0.05$). The combination with 2% CHX (G1) had the longest initial and final setting times ($P < 0.05$). All of the cements were alkaline and released calcium. [Tables 3 and 4] Show pH values and calcium release, respectively, from the materials studied at different experimental periods. In relation to pH values and calcium release, no statistical differences were detected among the groups, in all experimental periods ($P > 0.05$).

Discussion

In this study, the effect of adding 2% CHX alone and 5% CaCl₂ + 2% CHX to GMTA on setting time, pH and calcium release and antimicrobial activity were evaluated. The 5% CaCl₂ + 2% CHX combination, when used as MTA vehicle increased the antimicrobial action, and reduced the final setting time of MTA. The physico-chemical properties of GMTA (pH and calcium release) were no changed compared to control groups.

The antimicrobial activity of MTA cement can be evaluated by the agar diffusion method.^[6,7] In the present study, the antimicrobial activity of GMTA with additives was evaluated against *E. faecalis* and *M. luteus*. In fact, *E. faecalis* seems to play a significant role in the etiology of persistent periradicular lesions and has been used in numerous studies of antibacterial properties.^[17] *M. luteus* was utilized in the present study as control to *E. faecalis*.^[18]

The addition of 2% CHX did not increase the antimicrobial activity of GMTA. Holt *et al.*^[8] Observed that gray and white MTA cements mixed with 2% CHX showed significantly larger zone of inhibition of *E. faecalis* than MTA mixed with water. A possible explanation to this difference in the results could be the different strains of *E. faecalis* used in the studies. ATCC 29212 strains are originally isolated from urine, but used in standard antimicrobial

Table 1: Means and standard deviations of the inhibition zones (in mm)

	G1	G2	G3
<i>E. faecalis</i>	14.0 (0.0) ^a	15.0 (0.0) ^a	16.0 (0.0) ^b
<i>M. luteus</i>	22.0 (0.0) ^a	27.0 (0.0) ^{ab}	28.0 (0.0) ^b

GMTA=Gray mineral trioxide aggregate, CHX=Chlorhexidine digluconate, The values were expressed as the mean of four repetitions. ^{ab}Different letters indicate statistically significant differences on the same line ($P < 0.05$). G1 - GMTA+water, G2 - GMTA+2% CHX, G3 - GMTA+2% CHX+5% CaCl₂

Table 2: Means, standard deviations and statistical comparison of initial and final setting times (in minutes) for each combination evaluated

	G1	G2	G3
Initial setting	8.0 (1.0) ^a	16.0 (2.0) ^a	4.0 (1.0) ^b
Final setting	63.0 (2.0) ^a	95.0 (3.0) ^b	23.0 (1.0) ^c

GMTA=Gray mineral trioxide aggregate, CHX=Chlorhexidine digluconate, The values were expressed as the mean of four repetitions. ^{ab}Different letters indicate statistically significant differences on the same line ($P < 0.05$). G1 - GMTA+water, G2 - GMTA+2% CHX, G3 - GMTA+2% CHX+5% CaCl₂

Table 3: Means and standard deviations of the pH values of the materials studied at different experimental periods

	G1	G2	G3
24 h	9.65 (0.68)	9.48 (0.38)	10.02 (0.64)
7 d	9.78 (0.28)	9.84 (0.04)	9.77 (0.04)
14 d	10.09 (0.17)	10.10 (0.15)	10.17 (0.19)
28 d	10.32 (0.38)	10.54 (0.36)	10.57 (0.25)

GMTA=Gray mineral trioxide aggregate, CHX=Chlorhexidine digluconate, no statistically significant differences were observed between the experimental groups, in same period ($P > 0.05$). G1 - GMTA+water, G2 - GMTA+2% CHX, G3 - GMTA+2% CHX+5% CaCl₂

Table 4: Means and standard deviations of the calcium release (mg/l) values from the materials studied at different experimental periods

	G1	G2	G3
24 h	17.56 (2.37)	22.03 (5.61)	22.60 (5.46)
7 d	13.08 (0.86)	13.48 (1.49)	13.42 (0.98)
14 d	13.52 (2.09)	13.18 (2.50)	12.42 (2.10)
28 d	17.26 (6.28)	16.56 (3.94)	21.17 (6.86)

GMTA=Gray mineral trioxide aggregate, CHX=Chlorhexidine digluconate, no statistically significant differences were observed between the experimental groups, in same period ($P > 0.05$). G1 - GMTA+water, G2 - GMTA+2% CHX, G3 - GMTA+2% CHX+5% CaCl₂

assays.^[18,19] In another study, the authors used ATCC 4082 which was originally isolated from root canals of a pulpless tooth.^[20]

Chlorhexidine is active against a wide range of microorganisms.^[21] On the other hand, when the mixture of 5% CaCl₂ + 2% CHX was used, a significant increase in the antimicrobial activity of gray and white MTA cements was observed. Kuruvilla and Kamath^[11] observed that the use of NaOCl and CHX combined within the root canal produced the greatest percentage reduction of post-irrigant positive cultures

due to the formation of chlorhexidine chloride, which increases the ionizing capacity of the CHX molecule. The possible reason for this could be due to the following reactions: (a) CHX is a base capable of forming salts with a number of organic acids; (b) sodium hypochlorite is an oxidizing agent capable of oxidizing the gluconate part of CHX into gluconic acid. The chlor-groups might be added to the guanidine component of the chlorhexidine molecule, thereby forming "chlorhexidine chloride." If this were to happen, it would increase the ionizing capacity of the chlorhexidine molecule and the solution would incline towards an alkaline pH. Also, CaCl₂ dissociates into Ca⁺ and Cl⁻ ions, which could possibly react with CHX in similar way to that described by these authors, thus enhancing the antimicrobial action of MTA with chlorhexidine against both *E. faecalis* and *M. luteus*.

The setting time can be measured using a penetration test, with the help of gilmore needle.^[15] In the present study, the initial and final setting times were recorded when the stainless steel arm of the gilmore apparatus failed to make an indentation in the MTA. In apical surgery, MTA is placed against the tissue and is subject to washout by blood flow if special care is not taken.^[10] Therefore, a shorter setting time and an adequate adaptation in retrofilling cavity are desirable.^[1,22]

In the present study, 5% CaCl₂ + 2% CHX (G3) group had the shortest final setting time (23 min for GMTA), which was also significantly shorter than that of MTA with water (63 min for GMTA). Bortoluzzi *et al.*^[14] Observed initial and final setting times of 12 and 48 min for angelus white MTA, respectively, and when associated with 5% CaCl₂ initial and final setting times were of 6 and 31 min, respectively. However, it was not possible to make a real comparison between our results and literature because of the absence of studies assessing the effect of the association between 5% CaCl₂ + 2% CHX and MTA.

Addition of 2% CHX to GMTA resulted in an increase in the setting times (16 and 95 min, respectively, for initial and final setting times) compared to GMTA + water (8 and 63 min, respectively, for initial and final setting times). These results are consistent with Kogan *et al.*^[10] Which observed that MTA mixed with 2% CHX gel resulted in increased setting time.

The methodology used for evaluation of pH and calcium release consisted in filling standardized polyethylene tubes (0.5 cm length × 1.0 mm internal diameter) with the mixtures to be tested and immersing them in deionized water. After specific periods, pH was determined with pH meter and calcium release measured with atomic absorption spectrophotometer. Similar methods were employed in other studies, but with different tube dimensions both in length and inner diameter as well as different times of immersion in deionized water.^[3,15,16] The results showed that all mixtures were alkaline and released calcium, with no statistical difference between groups, regardless of the period of analysis or additives used. This showed that the addition of 2% CHX or 5% CaCl₂ + 2% CHX did not interfere with pH and calcium release of angelus MTA. Bortoluzzi *et al.*^[5] Found that the presence of 10% CaCl₂ increased the pH of angelus white MTA 30 min after manipulation. Moreover, these authors observed that products with CaCl₂ release more calcium than

pure materials within 24 h, but in our study CaCl₂ concentration was lower (5%), being first mixed in 1:1 (v/v) with 2% CHX and then added to GMTA cement. Further studies are needed for evaluation of the interference of such combinations with biological and other physico-chemical properties.

The addition of 2% CHX did not change pH, calcium release, or antimicrobial activity of GMTA, but increased the setting time of the cement. The association of 5% CaCl₂ + 2% CHX (1:1, v/v) reduced the setting time and enhanced the antimicrobial activity of GMTA without changing pH and calcium release.

Clinical significance

To solving endodontic accidents endodontic is recommended to use MTA-based cements. However, some its physico-chemical properties are questionable, such as large setting time and pH and calcium release. In the present study, a purpose for modified the MTA vehicle, to increase its properties, were evaluated.

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