Restor Dent Endod. 2020 Nov;45(4):e50
https://doi.org/10.5395/rde.2020.45.e50
pISSN 2234-7658·eISSN 2234-7666

Restorative Dentistry & Endodontics RDE

Research Article

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Incorporation of amoxicillin-loaded microspheres in mineral trioxide aggregate cement: an *in vitro* study

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Received: Mar 8, 2020
Revised: May 11, 2020
Accepted: May 26, 2020

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Conflict of Interest
No potential conflict of interest relevant to this article was reported.

Author Contributions
Conceptualization: Leitune VCB, Ogliari FA, Collares FM; Formal analysis: Bohns FR,

ABSTRACT

Objectives: In this study, we investigated the potential of amoxicillin-loaded polymeric microspheres to be delivered to tooth root infection sites via a bioactive reparative cement.
Materials and Methods: Amoxicillin-loaded microspheres were synthesized by a spray-dray method and incorporated at 2.5% and 5% into a mineral trioxide aggregate cement clinically used to induce a mineralized barrier at the root tip of young permanent teeth with incomplete root development and necrotic pulp. The formulations were modified in liquid:powder ratios and in composition by the microspheres. The optimized formulations were evaluated *in vitro* for physical and mechanical eligibility. The morphology of microspheres was observed under scanning electron microscopy.
Results: The optimized cement formulation containing microspheres at 5% exhibited a delayed-release response and maintained its fundamental functional properties. When mixed with amoxicillin-loaded microspheres, the setting times of both test materials significantly increased. The diametral tensile strength of cement containing microspheres at 5% was similar to control. However, phytic acid had no effect on this outcome ($p > 0.05$). When mixed with modified liquid:powder ratio, the setting time was significantly longer than that original liquid:powder ratio ($p < 0.05$).
Conclusions: Lack of optimal concentrations of antibiotics at anatomical sites of the dental tissues is a hallmark of recurrent endodontic infections. Therefore, targeting the controlled release of broad-spectrum antibiotics may improve the therapeutic outcomes of current treatments. Overall, these results indicate that the carry of amoxicillin by microspheres could provide an alternative strategy for the local delivery of antibiotics for the management of tooth infections.

Keywords: Aggregate trioxide mineral; Microspheres; Amoxicillin; Dental materials; Endodontics

Genari B, Dornelles NB Júnior, Collares FM; Investigation: Bohns FR, Leitune VCB, Genari B, Dornelles NB Júnior, Guterres SS, Ogliari FA, Collares FM; Project administration: Ogliari FA, Collares FM; Resources: Ogliari FA, Collares FM; Supervision: Leitune VCB, Ogliari FA, Collares FM; Visualization: Bohns FR, Garcia IM, Melo MAS, Collares FM; Writing - original draft: Bohns FR, Garcia IM, Collares FM; Writing - review & editing: Melo MAS, Collares FM.

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INTRODUCTION

Bacterial pathogens can reach the dental pulp, compromising the inner section of a tooth that contains the nerve tissue and blood vessels [1]. As a result of carious lesions or tooth fractures, bacteria gain entry into the pulp of the tooth and can be sheltered into dentinal tubules and root canal [2]. These microorganisms and their byproducts are responsible for initiating/maintaining a periapical inflammatory process of the pulp tissue that often become necrotic [3]. As the infection progresses, bacteria spread down the root, and through the apical foramen, reach the apex of the tooth root [4]. In cases of tooth infections in young patients, the affected tooth can be immature with incompletely formed roots. The treatment that met the standard of care for infected immature teeth relies on the use of a specific reparative material to neutralize the bacteria and to stimulate the root end closure process by forming a mineralized apical barrier [5,6].

The failure to induce root-end closure (apexification) is often caused by the persistence of microorganisms in the root canal system [7]. Mineral trioxide aggregate (MTA)-cements are Portland-like cement compounded by tricalcium silicate, tricalcium aluminate, tricalcium oxide, silicate oxide, and bismuth oxide [8]. This class of material has outstanding bioactivity with substantial calcium ions release and satisfactory performance as sealing material for root canal treatment [9]. Regrettably, most sealing materials, including MTA-based root canal sealers, lack of substantial antibacterial properties [10]. Some of them that were found to have bacteriostatic behavior, only retain this property for few days or lose this quality after the setting of the material [11,12]. As these materials have intimate contact with the root canal structures and tissues, it would be beneficial if sealing materials retained their antibacterial properties.

Polymer-based drug delivery systems provide a robust technology platform to enable sustained, spatiotemporally controlled drug release [13]. Microspheres have long been used in drug delivery applications because of their managed release capabilities [14]. Microsphere-based strategies have attracted attention in Dentistry because these materials may provide controlled release of bioactive molecules to promote disinfection and consequent healing of surrounds tissues [15]. Microspheres are organic or inorganic, spherical, free-flowing particles ranging from 1 to 1,000 μm in diameter that may carry drugs or bioactive molecules [16]. In medicine, they have been extensively used in drug delivery/targeting applications mainly because of their ability to enhance the efficacy of the associated drug by providing both a large surface area–to–volume ratio for drug release and spatial and temporal control over release [17].

Poly-methacrylic acid-co-methyl methacrylates are copolymers of methacrylate and methacrylic acid, and its use to delayed drug delivery are well reported [18]. This class of synthetic polymers, as known as Eudragits, presents variations in their proportion of neutral, alkaline, or acid groups, resulting in different physicochemical properties, including controlled release [19]. Overall, β-lactam antibiotics are used as the first option for the treatment of endodontic infections [20]. Amoxicillin is a broad-spectrum antibiotic with lower rates of bacterial resistance and the risk of side effects in endodontics [21]. The addition of the antibiotics system with a delayed-release profile to MTA could be a promising approach to improve the treatment outcomes (**Figure 1**).

Based on it, the present study investigated a simple and effective approach for the incorporation of amoxicillin-loaded polymeric microspheres into a modified formulation of bioactive cement and evaluated the resulting drug release profile and the effect on clinically relevant physical and mechanical properties.

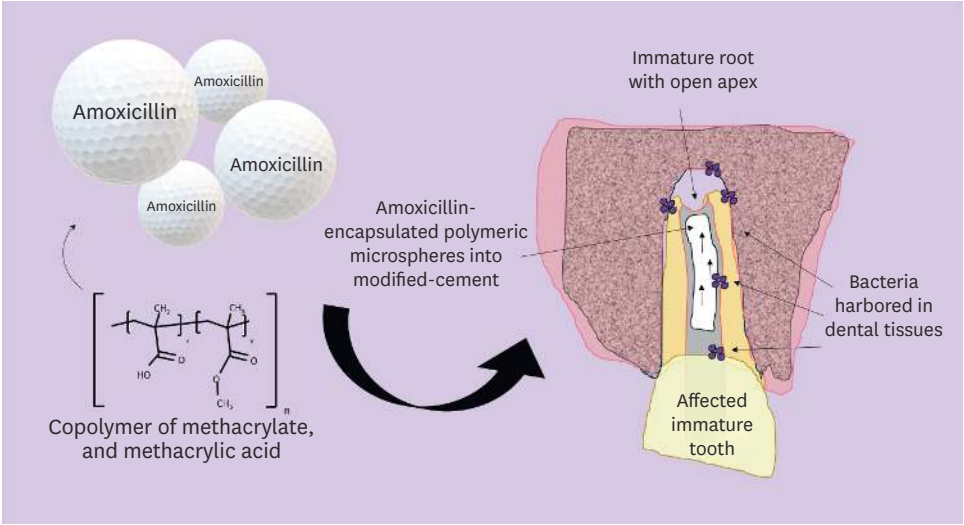

Figure 1. Illustrative scheme of a possible approach for the proposed bioactive dental cement with amoxicillin-loaded polymeric microspheres.

MATERIALS AND METHODS

Amoxicillin microspheres preparation

Microspheres loaded with amoxicillin (MS_{AMOX}) were prepared with poly(methacrylic acid-co-methyl methacrylate [Eudragit S100]), and trihydrate amoxicillin following a previous study [22]. Briefly, the polymer was first dissolved in acetone (200 mL), and subsequently, the amoxicillin was added under magnetic stirring at 25°C. The resultant solution was spray-dried using a Mini-Spray Dryer B-290 (Buchi, Flawi, Switzerland) coupled to a dehumidifier (B-292, Buchi) using the following parameters: feed pump rate of 5.0 mL min^{-1}, 100% aspiration, 0.7 mm nozzle, atomization air at 819 Lh^{-1}, and inlet temperature of 60°C with a resulting outlet temperature of approximately 40°C. **Figure 2** summarizes the synthesis route for the microspheres. Morphological characteristics of microspheres of amoxicillin were evaluated using scanning electron microscopy (SEM; JSM 6060, Jeol, Tokyo, Japan) with a voltage of 15 kW.

Morphological characterization

SEM was used to investigate the morphology and shell thickness of the prepared microspheres. After drying for 48 hours in a desiccator, the samples were then vacuum-coated with a thin layer of gold for 90 seconds to obtain scanning electron microscope images. These particles were examined using SEM at 5 kV.

Composition of mineral trioxide aggregate formulations

Synthetized MS_{AMOX} presented a mean size of 2.664 μm, d10 of 1.369 μm, d50 of 2.430 nm, and d90 of 4.315 μm according to a previous study [22]. To prepare the groups of MTA, MS_{AMOX} was mixed with the MTA at the weight percentages of 0%, 2.5%, and 5%. They were selected following favorable results of pilot tests. The MTA (WMTA Angelus, Angelus, Londrina, PR, Brazil) was used as parent material for the MS_{AMOX}'s incorporation. Preliminary studies were performed to tune the optimal liquid/powder ratio able to reach an acceptable homogenization of 5.0 wt% of MS_{AMOX}. The resulting homogeneity was achieved at a liquid/powder ratio of 1.16 mL/g. Based on these screening results, 2 formulations were used as

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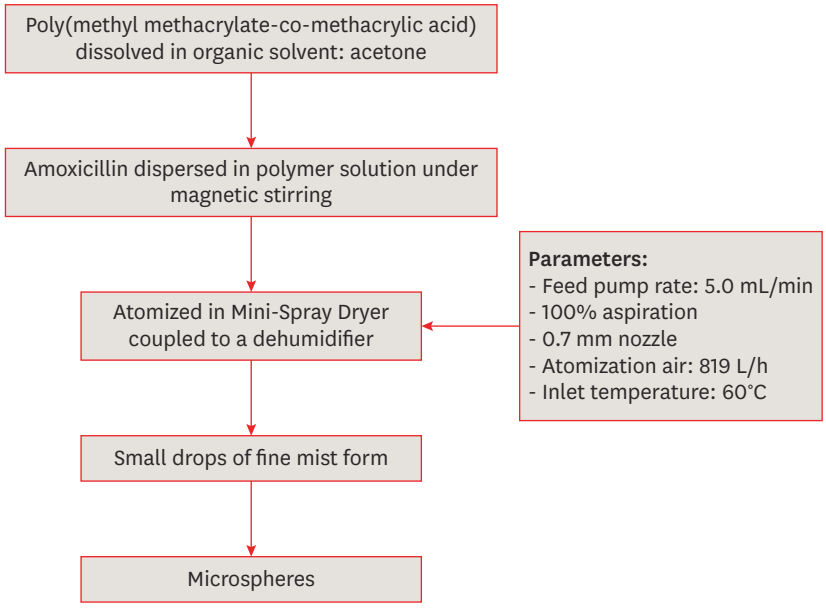

Figure 2. Schematic demonstration of the synthesis route for microsphere preparation by spray-dry method.

a control: 1) cement mixed with deionized water using the manufacturer's instruction at a liquid/powder ratio of 0.30 mL/g, ($Control_{ratio\ 0.3}$) and 2) cement mixed with deionized water at a liquid/powder ratio of 1.16 mL/g ($Control_{ratio\ 1.16}$). The experimental cements were prepared by mixing the tested MS_{AMOX} concentrations with deionized water at a liquid/powder ratio of 1.16. **Table 1** describes the chemical compositions of formulations assessed in this study.

Amoxicillin release profile

The group with the highest concentration of amoxicillin (MS_{AMOX} at 5%) was used to evaluate the drug release profile. To prepare the samples ($n = 4$), the cement was mixed and poured into cylindrical silicone molds measuring 5.5 mm of diameter and 2.5 mm of height. A polyester strip was placed on the top of each mold, and a load of 11 N was applied during 60 seconds to obtain a flat surface on each sample. All samples were stored in an incubator at 37°C for 7 days. The samples were put separately inside acetate membranes from Millipore (Millipore Corp., Darmstadt, Germany) with a pore size of 0.46 μm previously conditioned with deionized water for 30 minutes. The samples were immersed in 80 mL of simulated body fluid (SBF) prepared according to a previous protocol, followed by bath stirring (Quimiserv, EH4basic, Kika WERKE) [23]. After 2-, 6-, 12-, 24-, 48-, 72-, and 96 hours, 1 mL of SBF was collected and stored in Eppendorf tubes appropriately numbered; the same volume of SBF was replaced into the solution to avoid changes on fluid's concentration.

Setting time

The setting time of the test materials was determined by using the method recommended by ASTM C 266, also known as the Gillmore needle test with slight modifications [24]. To prepare

Table 1. Description of the composition from each group formulated in this study

Group	Description	Liquid-to-powder ratio
$Control_{ratio\ 0.3}$	Tricalcium silicate, dicalcium silicate, tricalcium aluminate, calcium oxide, bismuth oxide	0.30 mL/g
$Control_{ratio\ 1.16}$	Cement at the liquid-to-powder ratio of 1.16	1.16 mL/g
MS_{AMOX} at 2.5%	Cement at the liquid-to-powder ratio of 1.16 + 2.5% microspheres loaded with amoxicillin	1.16 mL/g
MS_{AMOX} at 5%	Cement at the liquid-to-powder ratio of 1.16 + 5% microspheres loaded with amoxicillin	1.16 mL/g

https://doi.org/10.5395/rde.2020.45.e50

the samples ($n = 3$) for setting time analysis, cylindrical silicone molds measuring 9 mm in diameter and 1.5 mm in height were used. The particles were incorporated into the mixture, and each cement was inserted into the silicone molds until it was filled. The excesses of the materials were removed, and a polyester strip was placed over each sample to obtain a flat surface. The samples were put in an incubator at 37°C and, after 120 seconds of the onset of the mixing, the initial mark was made using a Gillmore needle weighing 100 g and with a tip measuring 2 mm in diameter; this procedure was repeated at 60 seconds intervals and the values of initial setting time were recorded at the moment when the needle could not mark the sample surface.

Diametral tensile strength

The samples ($n = 3$) for diametral tensile strength (DTS) tests were prepared with cylindrical silicone molds measuring 9 mm in diameter and 1.5 mm in height. Each group was mixed and poured into the molds; the excess of the material was removed, and a polyester strip was put over the samples to secure a flat surface. All samples were allowed to set in an incubator at 37°C for 7 days. DTS of the samples was conducted on a universal testing machine Shimadzu (EZ-SX, Shimadzu, Japan) at a loading rate of 1 mm/min. DTS of the specimens were calculated using the relationship:

$$DTS = \frac{2P}{\pi bw}$$

Where: P is the maximum load (N), b is the diameter (mm), and w is the thickness (mm) of the measured specimen.

Statistical analysis

The software SigmaPlot, version 12.0 (Systat Software, Inc., San Jose, CA, USA) was used to data analysis. One way analysis of variance (ANOVA) and Tukey tests were used to evaluate the statistical significance data of setting time and DTS. Prematurely failure of specimens was included in the mean with the value of 0.18 MPa (mean value between zero and the minimum value observed in the study). A significant statistical difference was considered at $p < 0.05$.

RESULTS

Figure 3 shows SEM images of the microspheres fabricated by the spray-dry method. **Figure 3A** shows Spherical-shaped particles with a diameter ranging from 2 μm to 5 μm. The microspheres with its mashed shell indicate that the microspheres were not solid capsules but consist of a single internal cavity, which was occupied by amoxicillin. The non-spherical appearance of some of the particles can be due to the applied cutting force. The microspheres present irregular morphology and various size distribution. Some of these microspheres were also seen to be interconnected (arrow; **Figure 3B**). This is thought to have occurred as a result of liquid residue remaining between the microspheres and polymeric interconnection between the particles. The acetone subsequently acted as a solvent on minimal quantities of the shell surface, which is clearly indicated in **Figure 3C**.

The amoxicillin release profile of MS_{AMOX} mixed with the reparative cement at the weight percentages of 5% is shown in **Table 2**. The release profile demonstrated that the MS_{AMOX} effectively retarded the drug release after 7 days of storage and showed an approximately 17% of drug release after 96 hours of evaluation.

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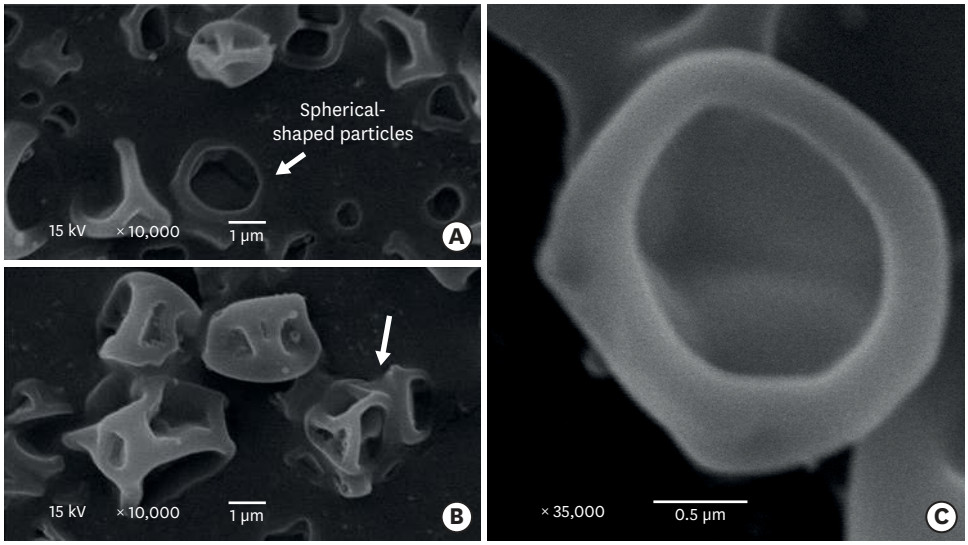

Figure 3. Scanning electron microscopy images of amoxicillin-loaded microspheres.

Table 2. Amoxicillin release profile of 5% microspheres loaded with amoxicillin

Collection periods (hr)	Amoxicillin release (%)
2	0.00
6	0.00
12	0.00
24	0.00
48	0.00
72	0.00
96	16.68

Figure 4 displays the initial setting time of the reparative cement with and without MS_{AMOX}. One way ANOVA and Tukey tests showed that the modification of power: liquid ratio of MS_{AMOX} significantly prolonged the initial setting time from 805 seconds to 1,433 seconds. When adding MS_{AMOX} at 2.5%, only slightly prolonged the initial setting time of 83 seconds was noted concerning $Control_{ratio\ 1.16}$. Increasing the concentration of MS_{AMOX} revealed an initial setting time of 1,417 seconds, similar to the $Control_{ratio\ 1.16}$. All the experimental formulations were statistically different from the $Control_{ratio\ 0.3}$ ($p < 0.05$).

The means and standard deviations of the compressive strength of the groups are shown in **Figure 5**. One way ANOVA and Tukey tests indicated statistically significant different values among groups. The compressive strength values of the $Control_{ratio\ 1.16}$ were significantly lower than the $Control_{ratio\ 0.3}$ ($p < 0.05$). Overall, the incorporation of MS_{AMOX} has improved the compressive strength results. MS_{AMOX} at 2.5% and MS_{AMOX} at 5% has shown an increase of 90.8% and 92.8%, respectively, in relation to $Control_{ratio\ 1.16}$. Comparing to the $Control_{ratio\ 0.3}$, the MS_{AMOX} at 5% has statistically similar.

DISCUSSION

In the present study, MS_{AMOX} were successfully synthesized and incorporated in a tuned formulation to grant delays in the amoxicillin release profile for the first time. With this approach, the prolonged antimicrobial effect during extended periods could increase

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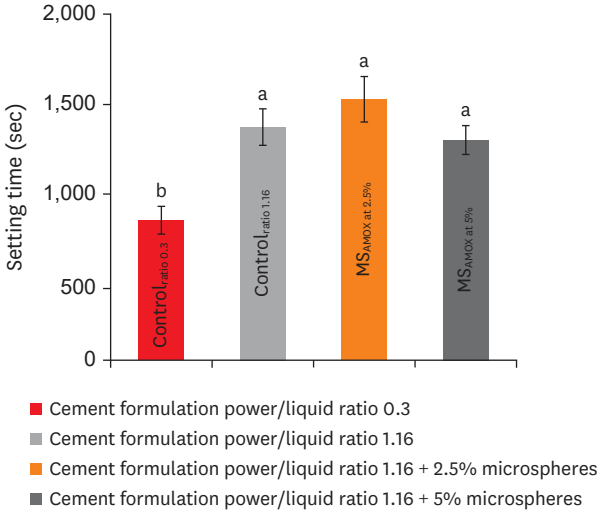

Figure 4. Setting times of tested cement formulations as the microsphere concentration increases. Values followed by the same letters are not significantly different ($p > 0.05$).

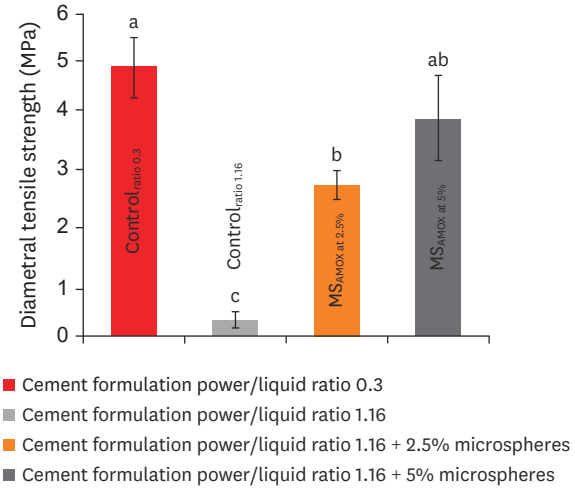

Figure 5. Diametral tensile strength (DTS) of the tested cement formulations according to microsphere concentration. Values followed by the same letters are not significantly different ($p > 0.05$).

the killing of remaining bacteria, which improve the healing tissue integrity and cellular function. Therefore, recent interests and significant efforts in preventing and actively treating tooth infections by directly targeting infection causative agents through direct application of antimicrobial agents either alone or loaded into dental materials. These have the advantage of overcoming challenges such as poor, locally delivered antibiotics [25]. Single-visit apexification using calcium silicate cements has been successfully used for the endodontic treatment of teeth with necrotic pulps and open apices [26].

Here, MS_{AMOX} effectively delayed the drug release when incorporated in the regenerative cement. At the measuring time of 96 hours, the amoxicillin release profile showed 16.68% of drug release but showed no release at previous measurements (**Table 2**). Similar results have been obtained by other researchers when using of acrylate-based polymers as a delayed carrier [26]. The microspheres obtained are monolithic particles retained in a porous or solid polymer matrix, differently from microcapsules that consist of a solid or liquid drug

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container, surrounded by a polymeric membrane [27,28]. The primary process of drug release from microspheres relays on diffusion, biodegradation, desorption of the adsorbed drug from the surface [14].

MS_{AMOX} can be prepared by solid-state reaction, co-precipitation, sol-gel method, and spray-dry routes [16]. In the current study, a facile method obtained the loaded microspheres using a Eudragit S100. The used copolymer solubilizes at pH 7. However, the alkaline medium induced by Ca^{2+} released from MTA-cements may delay its degradation, retarding the release of the drug [29,30]. The MTA's ability to increase the local pH makes this material very favorable as a vehicle for polymeric microspheres.

The setting behavior of MTA is driven by many variables such as the fineness of the particles, the water/powder ratio, temperature, humidity of application site, and the addition of substances [31]. As a Portland cement, MTA absorbs water. The water trapped in the cement mix and saturated by calcium hydroxide is then released to the environment [32]. High water content and the presence of amoxicillin microspheres may have affected the hydration mechanism of the used MTA-base cement, increasing the setting times of experimental groups. Fridland and Rosado [32] found that the physical properties of MTA changed when mixed with different water-to-powder proportions [33]. However, the amount of water incorporated in the mix is limited by its loss of consistency in the presence of excessive liquid. The modified MTA-base cement had a higher liquid-to-powder ratio in comparison with MTA, which needs more mixing liquid to achieve desired consistency to work. The targeted formulation of MS_{AMOX} at 5% presented suitable handing properties at the water-to-powder ratio of 1.16.

The liquid-to-powder ratio can also determine the mechanical properties of MTA-base cement. A higher liquid-to-powder ratio gives rise to an increasing degree of water pore volume, porosity, and solubility, which consequently can influence the mechanical characteristics of the cement. In our study, the incorporation of MS_{AMOX} has improved the compressive strength results. As amoxicillin has hydrophilic characteristics, its addition to the mixture may have contributed to absorbing water, remaining less unreacted water in the cement bulk, significantly increasing the strength of the targeted formulation of MS_{AMOX} at 5% [34]. Also, the smooth and round shape of microspheres works as a distributor of stress, which may contribute to the significant increase in mechanical property. The MS_{AMOX} at 5% presenting adequate strength can get the benefit of the humid environment because of the inherent hydrophilic nature. Further studies to investigate the *in vivo* behavior of MTA-base cement with synthesized MS_{AMOX} at 5% seem relevant.

CONCLUSIONS

In this work, we have tuned the formulation of a bioactive cement with polymeric microspheres to impart delayed release of antibiotics as a potential drug delivery system to treat local tooth infections. The resulting synthesis is facile, reproducible, and highly reliable. The incorporation of polymeric microspheres at the 5 wt% conveys delayed-release without significant detrimental effects of the physical and mechanical behavior of the material. The possibility of slowing the release of the antibiotics delivered at local sites of infection could find potential applications within dental materials that are in close contact with dental tissues.

https://doi.org/10.5395/rde.2020.45.e50

ACKNOWLEDGMENTS

The authors gratefully acknowledge Microscopy and Microanalysis Center (Federal University of Rio Grande do Sul) for the microscopy analyses and Angelus for providing the MTA used in this research. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior-Brasil (CAPES)—Finance Code 001—scholarship.

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