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ABSTRACT

The use of calcium hydroxide (CH) as an intracanal medicament for apexification is widespread. However, because of a short residence time in the root canal, the CH must be refreshed frequently, increasing the number of appointments required and leading to patient non-compliance. We hypothesized that a core-/shell-structured CH microsphere system would lead to sustained slow release of calcium and hydroxide ions of CH for long periods of time, eliminating the need for multiple visits for apexification. In this study, calcium hydroxide microspheres (CHMSs) with a core/shell structure were prepared by an emulsion method. The CHMS shell was composed of alginate, which was crosslinked by the Ca^{2+} released from the CH in the CHMSs. Therefore, this system provides a unique feedback loop that controls the release of ions from the CHMSs. The *in vitro* experiments from the root canals of extracted human teeth showed that the CHMSs had a sustained, slow release of Ca^{2+} , at a constant rate of approximately 2 to 3% *per* month from day one to the six-month endpoint of the experiment. After 6 months, $72.1 \pm 5.8\%$ of the total CH from the CHMSs remained in the root canals of the teeth, while only $46.9 \pm 10.9\%$ and $36.8 \pm 7.5\%$ remained from a commercial product (UltraCal®XS) and CH powder alone, respectively ($p < 0.01$). The pH of all of the formulations (CHMS, UltraCal® XS, and CH powder) in the extracted teeth never rose above 9 during the release period, indicating a buffering effect of the teeth. The core-/shell-structured CHMSs are, therefore, a promising delivery vehicle for the sustained slow release of Ca^{2+} and OH^- in the root canal.

KEY WORDS: alginate, core-/shell-structured microspheres, apexification, controlled release, extracted human teeth, dental pulp cavity.

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Endodontic Release System for Apexification with Calcium Hydroxide Microspheres

INTRODUCTION

Apexification is a procedure promoting the formation of a barrier that closes the open apex of an immature permanent tooth with a non-vital pulp so that the filling materials are contained within the root canal space (Farhad and Mohammadi, 2005). Among a variety of materials proposed for apical barrier formation, calcium hydroxide (CH) is the most widely accepted material because of its antimicrobial properties and ability to stimulate the formation of new bone (Mohammadi and Dummer, 2011). In an aqueous environment, CH dissolves into calcium and hydroxide ions. Calcium ions are thought to play an important role in the remineralization process (Ogata *et al.*, 2005; Narita *et al.*, 2010), while hydroxide ions generate an alkaline environment and are believed to be responsible for the antimicrobial action of CH (Çalt *et al.*, 1999; Farhad and Mohammadi, 2005).

Although CH pastes are the most widely used materials for apexification and apexogenesis, these materials possess some disadvantages. Typically, CH pastes require multiple visits to refresh the material and to confirm the formation of a new apical barrier, due to the length of time required for apical barrier formation (Abbott, 1998; Rafter, 2005). As with any dental treatment requiring multiple visits, non-compliance by patients is a concern. Tooth fracture after the long-term use of CH as a root canal dressing is also a drawback; some reports allude to the high pH of CH dressings as the cause of root fracture (Andreassen *et al.*, 2002).

In this study, we propose a slow-released CH microsphere (CHMS) for apexification of immature permanent teeth. We hypothesized that a core-/shell-structured CH microsphere system would promote the sustained slow release of calcium and hydroxide ions from CH for long periods of time, eliminating the requirement for multiple visits for apexification. The CHMSs are prepared by an emulsion technique that encapsulates the CH medicament in a carrier with an outer coating of alginate gel. Alginate has been shown to be biocompatible and biodegradable, and is used currently in the food industry as a thickener and emulsifying agent. Alginate is mildly crosslinked by divalent ions such as Ca^{2+} and forms gels without the use of harsh chemicals or specialized equipment (Martinsen *et al.*, 1989; Smidsrød and Skjåk-Bræk, 1990; Rowley *et al.*, 1999). In our CHMS system, the Ca^{2+} released from the CH will serve as a crosslinker for the alginate coating, therefore providing a unique feedback loop that controls the release of the ions from the CH. To further minimize the dissolution of CH, we added an oily carrier to form the CH core. The release profile of Ca^{2+} in extracted human teeth and pH values from the CHMS delivery system were compared with those of a commercial product (UltraCal® XS), CH powder alone, and no medicament.

Figure 1. Light-microscope images of representative CHMSs. (a) After 2 min of stirring. (b) After 10 min of stirring. The CHMS images show the calcium hydroxide powder (white) encapsulated in the alginate gel (translucent). The CHMSs were obtained by emulsion with oily CH in alginate solution (0.4% wt/v) with a mechanical stirring speed of 1,000 rpm. For observation purposes, relatively large CHMSs were selected for a clear view of the alginate outer layer. (c) The cumulative release profiles of Ca²⁺ from CHMSs (30 mg, prepared with stirring times of 2 and 10 min, respectively) in centrifuge tubes with PBS solution (1.0 mL).

MATERIALS & METHODS

CH and sodium alginate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Olive oil, UltraCal[®] XS calcium hydroxide paste (UC), and a calcium reagent set were purchased from Fisher Scientific (Fairlawn, NJ, USA). All the reagents were used as received, without further purification.

CHMS Preparation

The CH suspension was a 1:1 mixture (by mass) of CH powder and olive oil. In a typical CHMS synthesis, the CH suspension was injected with a 28G needle into a vigorously stirred (1,000 rpm) aqueous solution of alginate (0.4% wt/v). The emulsion was mixed for the period of time required to achieve the desired thickness of the alginate shell. The resulting alginate-encapsulated CHMSs were collected and sieved to separate them by size. For control groups, we prepared CH powder paste by mixing CH with distilled water at a weight ratio of 1:1, and the UltraCal[®] XS calcium hydroxide paste was used according to the manufacturer's instructions.

In vitro Release Experiments

The teeth were divided into 4 groups on the basis of the CH medicament as follows: Group 1, CHMS; Group 2, UC; Group

3, CHP; and Group 4, no medicament. Approximately 15 to 20 mg of the designated CH medicament was injected into each extracted tooth to fill the root canal. The Group 4 canals were left empty. The apical tip of each extracted tooth was left open, and then the tooth was propped on a polypropylene support, with only the root tip exposed to the medium (PBS-1X, 1 mL) in a tightly sealed 48-well plate to prevent evaporation. The total volume of media was removed and refreshed completely at pre-determined time-points. The pH of the release media samples was recorded, after which the liquid samples were stored at -20°C to prevent evaporation and then warmed to room temperature on a rotary shaker prior to analysis.

Evaluation of Release

The Ca²⁺ concentration of the release media was quantified by an indirect, colorimetric method of measuring the Ca²⁺ content by complexation with o-cresolphthalein (de Andrade Ferreira *et al.*, 2004). A calibration curve was constructed to obtain the molar absorptivity of the o-cresolphthalein with a Ca²⁺ standard and was used for quantification of the Ca²⁺ concentration. In a typical assay, 25 μL of the release media was added to 1 mL of the o-cresolphthalein solution. The solution was mixed, and the absorbance at 570 nm was recorded (Cary Win 50 UV-Vis spectrometer). To evaluate the OH⁻ release, we recorded the pH values for each group at room temperature, with the electrode submerged at least 1 inch in the solution.

The details for preparing the extracted teeth and calculating the total Ca²⁺ remaining in the extracted teeth are provided in the Appendix.

Statistical Analysis

The mean values ± standard deviations correspond to the mass percentage of Ca²⁺ and the pH levels (n = 6) in the different groups and were evaluated statistically by a one-way analysis of variance (ANOVA) followed by Tukey's test for individual comparisons of significant differences. Significance was set at *p* < 0.05.

RESULTS

The CHMSs were prepared by an emulsion method in a mechanically stirred diluted alginate solution. The CHMS formed a typical core/shell structure that encapsulated the CH in the alginate (Fig. 1). The size of the CHMS was controlled by the stirring speed. Smaller CHMSs were obtained with higher stirring speeds. Under the experimental conditions (10 min of stirring at 1,000 rpm), the majority of the CHMSs were in the range of 75 to 150 μm. The thickness of the alginate shell was controlled by adjustment of the stirring time of the alginate solution (Figs. 1a, 1b). Longer stirring times led to the formation of a thicker alginate outer layer, and therefore, the release of Ca²⁺ from the CHMSs was slower (Fig. 1c).

The total Ca²⁺ released and the cumulative Ca²⁺ released from the extracted tooth specimens in a six-month period are illustrated in Fig. 2. While Group 4 did show trace amounts of Ca²⁺ released from the teeth themselves, the total Ca²⁺ released was significantly less than from all of the other groups (*p* < 0.05) (Fig. 2a). Preliminary study also showed that CH mixed

Table. Summary of Calcium Ion Release and Average pH of Samples in Extracted Human Teeth after 6 months

Group	Medicament	Average pH in Extracted Teeth	Ca ²⁺ Released (mg)	Ca ²⁺ Released (%)	Ca ²⁺ Remaining (%)	Average pH in PPL Tubes
1	CHMS	7.58 (± 0.20)	4.09 (± 0.39)	27.9 (± 5.8)	72.1 (± 5.8)	7.80 (± 0.39)
2	UC	7.65 (± 0.14)	8.77 (± 1.81)*	53.1 (± 10.9)*	46.9 (± 10.9)*	8.52 (± 0.55)
3	CH	7.96 (± 0.30)	15.32 (± 2.07)*	63.2 (± 7.5)*	36.8 (± 7.5)*	8.10 (± 0.66)
4	No medicament	7.45 (± 0.02)	0.79 (± 0.12)*	N/A	N/A	7.45 (± 0.01)

* Indicates significant difference ($p < 0.05$) compared with CHMS.

with olive oil had a significantly higher Ca²⁺ release rate than the CHMS during the experimental period (data not shown). Therefore, only 3 groups (CHMSs, UC, and CHP) were evaluated in this study. In these groups, an initial burst release was observed in the first 8 hrs; however, the burst release in the CHP group was significantly higher than in the other 2 groups ($p < 0.05$) (Fig. 2b). For the first 4 days, the CHMS group displayed a Ca²⁺ release similar to that of the UC group; however, by day 10, the Ca²⁺ release from the UC group was significantly higher than from the CHMSs ($p < 0.05$). As seen in Fig. 2b, the release rate of the CHP and UC reached a plateau after 5 wks, and only 3.1 and 1.1%, respectively, of the total calcium in the controls was released during the last 4½ mos of the experiment. The CHMSs released Ca²⁺ consistently at a rate of approximately 2 to 3% *per mo* from day one to the six-month endpoint of the experiment (Fig. 2c). Overall, a total of $72.1 \pm 5.8\%$ of CH remained in the CHMSs at the end of 6 mos, while only $46.9 \pm 10.9\%$ ($p < 0.001$) and $36.8 \pm 7.5\%$ ($p < 0.001$) remained in the UC and the CHP, respectively (Table).

Fig. 3 illustrates the average pH values over time for each of the 4 medicament groups in the extracted teeth. In general, all of the groups with medicament (CHMS, UC, and CHP) showed overall increases in pH after 6 mos compared with day one. However, the pH of all of the groups was between 7 and 9 at all times. In the extracted tooth specimens at 6 mos, the average pH was 7.58 ± 0.20 , 7.65 ± 0.14 , 7.96 ± 0.30 , and 7.45 ± 0.01 for CHMSs, UC, CHP, and no medicament, respectively (Table). None of the average pH values for the specimens was significantly different in any individual comparisons ($p > 0.05$).

To examine the buffering effect of the teeth, we performed a parallel Ca²⁺ release experiment in polypropylene (PPL) tubes. Throughout the 6-month release measurement, the pH of the CHP and UC groups in the PPL tubes was high, fluctuating between 7.5 and 10 and averaging 8.52 ± 0.55 for UC and 8.10 ± 0.66 for CHP (Table). The CHMS group in the PPL tubes displayed a constant and mildly alkaline pH value, averaging

Figure 2. Colorimetric Ca²⁺ determination of different CH medicament groups tested in extracted human teeth ($n = 6$). The CHMSs were prepared by emulsion with oily CH in alginate solution (0.4% wt/v). (a) Total mass of Ca²⁺ released; (b) 5-week release profiles; inset shows the first 24-hour release profiles; (c) 6-month release profiles.

7.80 ± 0.39 . When the pH values of the PPL samples were compared with those of the same group from the extracted teeth, a significant difference was observed ($p < 0.05$, comparisons of the same group in PPL tubes vs. teeth), indicating the buffering effect of the teeth.

DISCUSSION

In our CHMS system, the Ca²⁺ released from the CH core served to crosslink the alginate in the outer layer of the CHMSs. The crosslinked alginate, in turn, controlled the release of the ions of the CH inside the CHMSs. Therefore, this core/shell structure provided an intelligent feedback loop to maintain the alginate coating, which thus controlled the release of the CH ions. This work is the first report of the use of alginate gel to control CH release. The size of the CHMSs determines their injectability and is controlled by the stirring speed, while the thickness of the alginate shell is controlled by the stirring time in the alginate solution. The release of chemicals encapsulated in alginate gels

Figure 3. *In vitro* pH values over time of different CH medicament groups in extracted human teeth. (a) At 2 wks, inset shows the first 24-hour pH value curves; (b) 6-month pH value curves.

is generally controlled by 2 mechanisms: (1) diffusion of the chemicals through the pores of the alginate network, and (2) degradation of the alginate network (Gombotz and Wee, 1998). Because the alginate gel constantly interacts with the Ca^{2+} released from the CH in the CHMSs, the alginate outer layer of the CHMSs is stable and does not degrade during the release process. Therefore, the release of the ions in the CHMSs is controlled by a diffusion mechanism. Since the diffusion rate and thickness of a hydrogel have an inverse relationship (Gombotz and Wee, 1998), increasing the thickness of the alginate outer layer leads to a slower release of Ca^{2+} from the CHMSs.

Adding olive oil to the CH core provides a layer of protection from the aqueous environment. Studies have shown that while aqueous solutions of CH rapidly increased the pH in tissues, oily CH suspensions produced a long-lasting gradual pH increase without irritation and were more effective than water-based vehicles at reducing microbial populations (Kozlovsky *et al.*, 1996; Kasaj *et al.*, 2006). A review of CH-delivery vehicles indicated that a relatively fast initial ion release is important after endodontic treatment (Fava and Saunders, 1999). The initial release profile of the CHMS group was similar to that of the

UC group under the experimental conditions ($p > 0.4$ for all time-points up to day 4). Therefore, the CHMSs were at least as effective in the first days after treatment as the UC, with the added benefit of controlling the rate of release for an extended period of time.

After an early plateau during the release, both the UC and CHP groups experienced a secondary burst release, which led to the use of 25 to 45% more of the CH in those groups compared with the CHMSs. Despite the fact that 37 to 47% of the original CH remained, the UC and CHP groups stopped releasing detectable quantities of Ca^{2+} at the four-month time-point, consistent with other aqueous/viscous formulations (Fava and Saunders, 1999). In contrast, the CHMSs consistently released Ca^{2+} at a rate of approximately 2 to 3% *per mo* up to the culmination of the experiment at 6 mos.

In the experiments where PPL tubes were used to mimic the root canal, the pH values of the CHP and UC groups were high, which were consistent with those of experiments performed under similar conditions (de Andrade Ferreira *et al.*, 2004). However, the CHMS group specimens in the PPL tubes displayed a constant and mildly alkaline pH, indicating the ability of the formulation to slow the diffusion of hydroxide. In the extracted tooth specimens, the buffering effect of the teeth/dentin kept the pH of all of the groups consistently in the range of 7.5 to 9 ($p < 0.05$, comparisons of same group in PPL tubes *vs.* teeth). The moderately alkaline pH of the samples in the extracted teeth throughout our study differed from another report that utilized viscous-aqueous polymers to deliver CH in extracted teeth and showed that all of the formulations reached a pH of ~10 or more after 1 mo (Ballal *et al.*, 2010). These contrasting results are likely due to differences in the experimental design. In their study, only a portion of the release media was removed at each time-point, allowing the OH^- to build up until the end of the experiment. The literature indicates that, while an initial alkaline environment can be favorable for mineralization (Fava and Saunders, 1999), long-term highly alkaline treatments are not beneficial and may increase the risk of tooth fracture (Andreasen *et al.*, 2002). The efficacy, antibacterial properties, and degradation products of the CHMSs will be the subjects of future studies determining the effectiveness of the CHMSs as an intracanal CH medicament for apexification.

In summary, we prepared core-/shell-structured calcium hydroxide microspheres by combining an oily vehicle and alginate outer coating method. Compared with commercial UltraCal[®] XS and calcium hydroxide powder, the core-/shell-structured microspheres maintained a sustained slower release of Ca^{2+} and persistent mildly alkaline pH values over 6 mos in extracted human teeth *in vitro*. The CHMSs, therefore, are a promising delivery vehicle of calcium hydroxide for apexification.

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