The Biological Performance of Calcium Hydroxide–loaded Microcapsules

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Abstract

Introduction: Calcium hydroxide ($Ca[OH]_2$) microcapsules were synthesized for use in controlled release. The aim of this study was to evaluate the cytotoxicity, antibacterial properties, and influence on gene expression of bone-related markers of 2 different formulas of $Ca(OH)_2$ microcapsules. **Methods:** Two formulas of $Ca(OH)_2$ microcapsules (A and B) were evaluated, and pure $Ca(OH)_2$ powder was used as a positive control. The shell material of formula A was pure EC, and the PLA/EC blend of 1:1 was used as the shell material for formula B. The MG63 cells/Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) were used to evaluate the cytotoxicity, and the colony-forming units of *Enterococcus faecalis* were monitored for the antibacterial effect. The relative messenger RNA expression of collagen I and osteocalcin was determined by real-time polymerase chain reaction. **Results:** Both formulas of the $Ca(OH)_2$ microcapsules showed no cytotoxicity in MG63 cells; however, the $Ca(OH)_2$ positive control did exhibit cytotoxicity. The antibacterial effect of the 2 microcapsule formulas lasted longer than the positive control, and formula A lasted longer than formula B. For both $Ca(OH)_2$ microcapsule formulas, the relative messenger RNA expression of collagen I and osteocalcin was prolonged and up-regulated. The time effects of the influence on messenger RNA expression of collagen I and osteocalcin were different between the 2 microcapsule formulas. **Conclusions:** $Ca(OH)_2$ microcapsules had prolonged antibacterial activity and prolonged the up-regulation of bone-related markers with reduced cytotoxicity. *(J Endod 2013;39:1030–1034)*

Key Words

Biological performance, calcium hydroxide, controlled release, microcapsules, sustained release

Calcium hydroxide ($Ca[OH]_2$) was introduced into dentistry by Hermann and occupies a prominent position as a versatile medicament in endodontics because of its biological properties (eg, the induction of hard-tissue deposition and antimicrobial activity) (1). The properties of $Ca(OH)_2$ medicament are mainly influenced by the vehicles used, either aqueous, viscous, or oily (2). The disadvantage of aqueous and viscous vehicles is the fast release of ions (1); however, the cytotoxicity, immunogenicity, low $Ca(OH)_2$ loading, and difficulty in removal limit the application of oily vehicles (2–5).

$Ca(OH)_2$ may show high cytotoxic effects when in direct contact with adjacent tissues because of high alkalinity caused by its fast release (6, 7). The rapid release of ions and low loading of $Ca(OH)_2$ also limit the antibacterial and biomineralization effects of $Ca(OH)_2$ medicaments.

The main function of $Ca(OH)_2$ is to provide antimicrobial activity. This activity in bacterial cells is probably caused by the following mechanisms (8):

1. Damage to the bacterial cytoplasmic membrane
2. Protein denaturation
3. DNA damage

In a previous study, Vitapex (a commonly used $Ca(OH)_2$ medicament with an oily vehicle; Neo Dental Chemical Products Co Ltd, Tokyo, Japan) had poor antibacterial performance compared with formulations containing 50%–60% $Ca(OH)_2$ with sterile saline as an aqueous vehicle (9).

With regard to biomineralization properties, collagen I (Col I) and osteocalcin (OCN) are the most representative osteoblastic markers and are widely used to evaluate bone formation and mineralization of biological mineralized material. The most abundant matrix protein in bones is Col I. Collagen contributes to the mechanical properties of bone and is necessary for calcification of the tissue. OCN is a gamma–carboxyglutamic acid–containing protein of bone, which has an affinity to hydroxyapatite and can prevent crystal growth (10). These are the most frequently used indicators of osteoblast differentiation and osteogenic properties (11–13).

In order to improve the biological performance of $Ca(OH)_2$ medicaments, controlled-release $Ca(OH)_2$-loaded microcapsules based on polylactic acid (PLA) and ethylcellulose (EC) were developed (14). These microcapsules prolonged the release of ions, which could be controlled by regulating the ratio of PLA/EC. When more EC was used as the shell material, the release was much slower (14). However, further studies are required to determine whether $Ca(OH)_2$ microcapsules influence the biological properties compared with $Ca(OH)_2$ powder.

In the present study, we evaluated the cytotoxicity, antibacterial activity, and influence on gene expression of bone-related markers of 2 different formulas of

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$Ca(OH)_2$-loaded microcapsules. Pure $Ca(OH)_2$ powder was used as a positive control. The hypothesis for this study was that $Ca(OH)_2$ microcapsules would prolong the antibacterial effects and up-regulation of bone-related markers with reduced cytotoxicity.

Materials and Methods

In the present study, 2 formulas of PLA/EC (w/w ratio) microcapsules were evaluated: formula A (ie, pure EC) and formula B (ie, PLA/EC 1:1). The $Ca(OH)_2$ microcapsules were prepared using the phase-separation technique, and the preparation details were described in our previous study (14). In that study, the morphology and composition, particle size distribution, glass transition temperature, drug loading, and encapsulation efficiency were characterized (14). In the study evaluating the *in vitro* release profile, it took 456 and 264 hours for formula A and B, respectively, to release 90% of their total $Ca(OH)_2$ content (14). The release kinetics of ions by the microcapsules fitted a first-order model well (14). Figure 1*A* through *D* shows the morphology, the core-shell heterostructure, and the size distribution of the microcapsules used in this study (14).

Cytotoxicity Evaluation

The human osteosarcoma cell line MG63 was purchased from the American Type Culture Collection and cultured as recommended. The cells were cultured with Dulbecco modified Eagle medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 100 μg/mL streptomycin, and 100 U/mL penicillin in a CO_2 incubator (Thermo, Waltham, MA) at 37°C. After reaching 80% confluence, the confluent cells were digested with 0.25% trypsin and 0.05% EDTA (Gibco) and subcultured in 96-well plates (2.5×10^3 cells/100 μL medium in each well) for another 12 hours to allow attachment. The medium was then removed, and 100 μL fresh medium with medicament ($Ca[OH]_2$ microcapsules 1500 μg/mL medium, $Ca[OH]_2$ powder 1000 μg/mL medium) was added. In the negative control, the cells were cultured in medium without medicament. Five wells were used for each group. In order to verify the effect of reduced cytotoxicity of microcapsules, a higher concentration of microcapsules was selected in the present study. In theory, the effective concentration of $Ca(OH)_2$ in the 2 microcapsule groups was approximately 1200 μg/mL (1500 μg/mL $\times$ 80%) according to drug loading of the $Ca(OH)_2$ microcapsules (approximately 80%) (14). The concentration of $Ca(OH)_2$ in the 2 microcapsule groups (1200 μg/mL) was higher than that in the positive control group (1000 μg/mL). After incubating the cells for 0, 1, 3, 5, and 7 days, respectively, cell viability was determined using the Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan). Day 0 cells were cells that were not incubated with medium containing medicaments. The results of day 0 were to show that the cells were dispersed uniformly in each well. In the experiment of cytotoxicity evaluation, the results of day 0 were used as baseline. The cells were washed with phosphate-buffered saline (PBS) twice and fresh culture medium (100 μL), and then CCK-8 reagent (10 μL) was added to each well. After incubation for 2 hours, the optical density (OD) of each well was measured using the Elx808 microplate reader (Bio-Tek, Winooski, VT) at 450 nm with a reference wavelength of 630 nm.

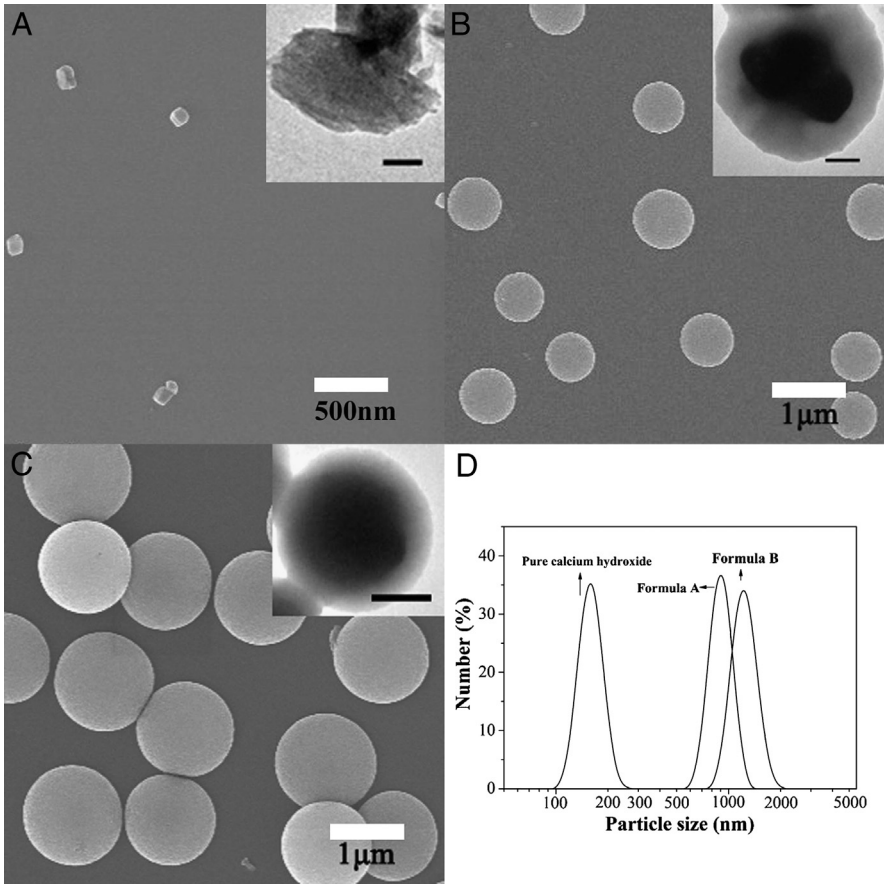

Figure 1. Scanning electron microscopic images, TEM images (*Inset*), and size distribution of microcapsules used in this study (14). (*A*) Pure $Ca(OH)_2$ (the bar in the *Inset* represents 50 nm), (*B*) formula A (the bar in the *Inset* represents 100 nm), (*C*) formula B (the bar in the *Inset* represents 500 nm), and (*D*) particle size distributions of $Ca(OH)_2$ and microcapsules.

Antibacterial Assessment

Enterococcus faecalis (American Type Culture Collection 29212) was used in this study. Isolated pure cultures of *E. faecalis* grown on 5% defibrinated sheep blood–brain heart infusion (BHI) (Becton, Dickinson and Co, Franklin Lakes, NJ) agar plates were suspended in BHI broth. The suspension of *E. faecalis* was adjusted to a turbidity of 3×10^8 colony-forming units (CFUs)/mL. Twenty microliters of bacterial suspension and 1.5 mL BHI broth were added to each well of a 24-well plate. In this experiment, a Transwell insert (0.4-μm filter; Millipore, Billerica, MA) was used to hold 15 mg medicament (2 $Ca[OH]_2$ microcapsule groups and pure $Ca[OH]_2$ powder) in the well, and 0.2 mL BHI broth was added simultaneously. The inserts without medicaments were added to the wells of the negative control group. After incubation for 24 hours, the Transwell inserts in the 4 groups were moved into new wells of the 24-well plate containing 20 μL fresh bacterial suspension and 1.5 mL fresh BHI in each well. Before being reimmersed in the new well, the outside surface of the Transwell insert was washed with PBS. The broth left in the well was diluted appropriately and plated on BHI agar plates. The plates were incubated at 37°C for 24 hours, and the surviving colonies were then counted to determine the number of CFUs. This procedure was repeated, and the antibacterial activity of the medicaments was assessed continuously for 7 days. All experiments were performed in triplicate.

Influence on Gene Expression of Bone-related Markers

The Medicament and Cell Preparation. A Transwell insert (0.4-μm filter) of a 6-well plate was used to hold the medicament. The Transwell insert held 15 mg $Ca(OH)_2$ medication (2 $Ca[OH]_2$ microcapsule groups and pure $Ca[OH]_2$ powder), and 4 mL culture medium were prereleased in the 6-well plate. Each well contained 6 mL culture medium, which was changed every other day. The Transwell insert holding prereleased (for 14, 7, 5, 3, 1, and 0 days) $Ca(OH)_2$ medicaments were used for the experiments.

MG63 osteoblast-like cells were used for these experiments. The cells were cultured in culture flasks (Corning, Corning, NY), and the culture medium was changed every 2 days. After reaching 80% confluence, the cells were digested and transferred to the 6-well culture plate. The inserts holding prereleased medicaments were placed in the wells containing cells and cultured for 3 days, respectively, and further experiments detailed later were completed. All experiments were performed in triplicate.

Quantitative Real-time Polymerase Chain Reaction. The cells in the well were washed twice with PBS and harvested using Trizol reagent (Invitrogen, Carlsbad, CA) for the detection of OCN and Col I messenger RNA (mRNA) expression using real-time polymerase chain reaction. RNA was extracted using Trizol according to the manufacturer's instructions and was reverse transcribed to complementary DNA using a reverse transcription kit (Fermentas, Vilnius, Lithuania). Real-time PCR reactions were performed using Faststart Universal SYBR Green Master (Rox; Roche, Basel, Switzerland) in an ABI 7500 real-time Thermocycler (Applied Biosystems, Foster City, CA). Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was used as an internal control. The sequences of the primers were as follows: Col I forward primer, 5′-ATGGGAGGAGAGCGTGTG-3′; Col I reverse primer, 5′-GAGGTCGGAGAGCAGAGG-3′; OCN forward primer, 5′-CACTCCTCGCCC-TATTGGC-3′; OCN reverse primer, 5′-CCCTCCTGCTTGGACACAAAG-3′; GAPDH forward primer, 5′-GAAGGTGAAGGTCGGAGTC-3′; GAPDH reverse primer, 5′-GAAGATGGTGATGGGATTTC-3′. The data were analyzed using SDS software (Applied Biosystems, Inc, Carlsbad, CA) according to the manufacturer's instructions and presented as relative mRNA levels calculated by the equation $2^{-\Delta Ct}$ (ΔCt = Ct of target gene minus Ct of GAPDH) (15).

Statistical Analysis

Data were analyzed using SPSS 11.5 software (SPSS, Inc, Chicago, IL) (1-way analysis of variance). For all tests, statistical significance was accepted for P values lower than .05.

Results

Cytotoxicity Evaluation

As shown in Figure 2*A*, there was no statistical difference in the cytotoxicity among the 4 groups on days 0 and 1 ($P > .05$). The ratio of OD values relative to the negative control of the positive control group were lower than those of the negative control group on days 3, 5, and 7 ($P < .01$). There was no difference between the 2 $Ca(OH)_2$ microcapsule groups and the negative control group ($P > .05$). Although the $Ca(OH)_2$ concentrations in the 2 microcapsule groups were higher than the positive control group according to drug loading (approximately 80%) (14), the ratio of OD values relative to the negative control of the 2 microcapsule groups was higher than those of the positive control group on days 3 ($P < .01$), 5 ($P < .05$), and 7 ($P < .01$).

Antibacterial Evaluation

The antibacterial activity results are shown in Figure 2*B*. On days 1 and 2, the number of CFUs in the 2 $Ca(OH)_2$ microcapsule groups and the positive control group were lower than that for the negative control group ($P < .01$). On day 1, the number of CFUs in the 2 $Ca(OH)_2$ microcapsule groups was higher than the positive control group ($P < .01$). On day 3, the number of CFUs in the 2 $Ca(OH)_2$ microcapsule groups was lower than those in the positive ($P < .01$) and negative control groups ($P < .01$). On day 4, the number of CFUs in formula A was lower than that in the negative and positive control groups ($P < .01$). On day 5, the number of CFUs in formula A was lower than the positive control group ($P < .05$). On days 6 and 7, there was no difference between the 4 groups ($P > .05$). According to these results, the positive control group had no antibacterial ability on day 3, formula B exhibited antibacterial activity up to day 3, and formula A exhibited antibacterial activity up to day 4.

Influence on Gene Expression of Bone-related Markers

The relative mRNA expression of Col I and OCN is shown in Figure 2*C* and *D*, respectively. Gene expression in the positive control group was not detected with inserts prereleased for 0 day. The relative mRNA expression of Col I in the 2 $Ca(OH)_2$ microcapsule groups was higher than that of the negative control group with inserts prereleased for 0 ($P < .01$), 1 ($P < .01$), 3 ($P < .01$), 5 ($P < .01$), 7 ($P < .05$), and 14 ($P < .05$) days. mRNA expression of Col I in the formula A group was higher than that in the positive control group with inserts prereleased for 3 ($P < .05$), 5 ($P < .01$), and 7 ($P < .01$) days.

The relative mRNA expression of OCN in the positive control group was higher than that in the negative control group with inserts prereleased for 1 day ($P < .01$). The mRNA expression of OCN in the formula B group was higher than that in the negative control group with inserts prereleased for 0 ($P < .05$) and 1 day ($P < .01$). The mRNA expression of OCN in the formula A group was higher than that in the negative control group with inserts prereleased for 3 ($P < .05$) and 5 days ($P < .01$). The mRNA expression of OCN in the formula A group was higher than that in the positive control group with inserts prereleased for 3 ($P < .05$) and 5 days ($P < .01$). There was no statistical difference in mRNA expression between the 4 groups with inserts prereleased for 7 and 14 days ($P > .05$).

Discussion

In the present study, the hypothesis had been verified that $Ca(OH)_2$ microcapsules prolonged antibacterial activity and increased the

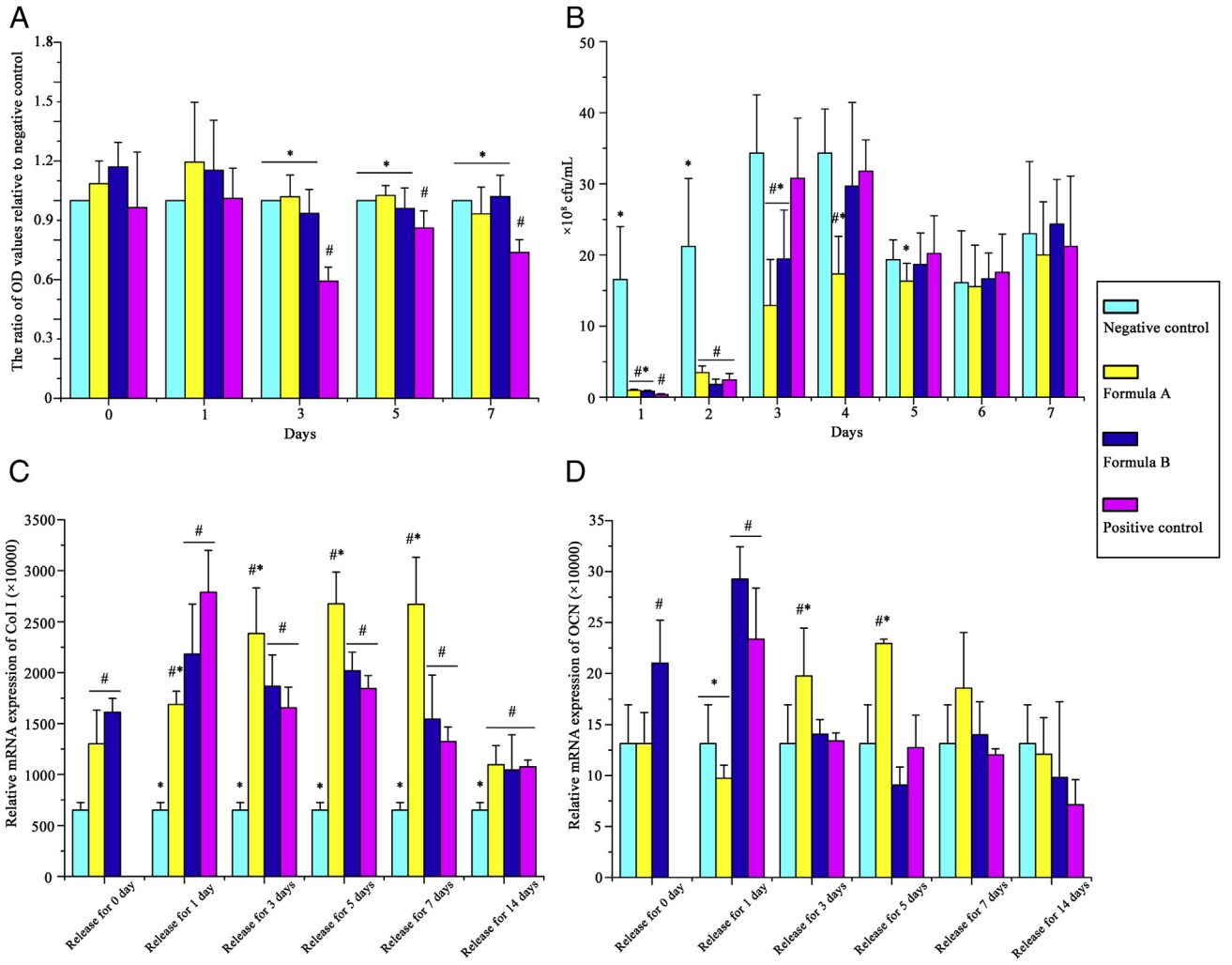

Figure 2. The biological performance of 2 $Ca(OH)_2$-loaded microcapsules. The results represent the mean ± standard deviation. *$P < .05$ versus the positive control group. #$P < .05$ versus the negative control group. (*A*) Cytotoxicity evaluation of $Ca(OH)_2$ microcapsules and pure $Ca(OH)_2$ in MG63 cells. The positive control group had cytotoxicity on days 3, 5, and 7, whereas the 2 formulas of $Ca(OH)_2$ microcapsules had no cytotoxicity. (*B*) The antibacterial activity of $Ca(OH)_2$ microcapsules and pure $Ca(OH)_2$. The positive control group exhibited antibacterial activity on days 1 and 2, formula B exhibited antibacterial activity up to day 3, and formula A exhibited antibacterial activity up to day 4. The 2 formulas of $Ca(OH)_2$ microcapsules could prolong the antibacterial effect. (*C*) The relative mRNA expression of Col I with inserts released for different days. Gene expression in the positive control group was not detected with inserts prereleased for day 0. The relative mRNA expression of Col I in the 2 $Ca(OH)_2$ microcapsule groups was higher than that of the negative control group with inserts prereleased for days 0 ($P < .01$), 1 ($P < .01$), 3 ($P < .01$), 5 ($P < .01$), 7 ($P < .05$), and 14 ($P < .05$). mRNA expression of Col I in the formula A group was higher than that in the positive control group with inserts prereleased for 3 ($P < .05$), 5 ($P < .01$), and 7 ($P < .01$) days. (*D*) The relative mRNA expression of OCN with inserts released for different days. Gene expression in the positive control group was not detected with inserts prereleased for 0 days. The relative mRNA expression of OCN in the positive control group was higher than that in the negative control group with inserts prereleased for 1 day ($P < .01$). The mRNA expression of OCN in the formula B group was higher than that in the negative control group with inserts prereleased for 0 ($P < .05$) and 1 day ($P < .01$). The mRNA expression of OCN in the formula A group was higher than that in the negative control group with inserts prereleased for 3 ($P < .05$) and 5 days ($P < .01$).

expression of biological mineralization factor with reduced cytotoxicity *in vitro* compared with pure $Ca(OH)_2$. Biodegradable and biocompatible microparticles and nanoparticles could get sustained, controlled, and targeted delivery of drugs in medical areas in addition to reducing the cytotoxicity of medicaments in some areas of medicine. In the field of endodontic studies, the chitosan nanoparticles were prepared to inhibit biofilm formation within the sealer-dentin interface (16). Also, it had been verified that nanoparticles would have considerable potential advantages in root canal disinfection (17). In this study, pure $Ca(OH)_2$, which was compared with $Ca(OH)_2$, encapsulated by EC/PLA showed improved biocompatibility, antibacterial properties, and biomineralization activity.

Cytotoxicity can be determined with reliability and reproducibility (18). The CCK-8 test is one of the cytotoxicity evaluation methods (19, 20), and the principle of CCK-8 is to test the dehydrogenase activity in cells of living cells. In order to avoid the heterogeneity of osteoblasts or odontoblasts derived from primary culture, whose function might be influenced by cell source and cell generation, human osteosarcoma cells were used in the present study. MG63 is a bone cell line with remodeling capability. It was widely used in studies related with the cytotoxicity of materials and bone marker expression (21, 22). The cytotoxicity evaluation findings were in accordance with previously published studies (7, 23). For example, Costa et al (7) showed that $Ca(OH)_2$ solutions applied to cultured cells decreased cell metabolic

activity by 29.4%. It was believed that the high alkalinity of the culture medium caused by $Ca(OH)_2$ dissolution had an adverse effect on the culture and growth of cells. Narita et al (23) reported that osteoblasts were unable to survive in a medium containing high doses of $Ca(OH)_2$ (2.5 mg/mL); however, osteoblasts cultured with low doses of $Ca(OH)_2$ (ie, 0.25 and 0.025 mg/mL) were similar to those cultured without $Ca(OH)_2$. The reason that 2 $Ca(OH)_2$ microcapsule formulations showed no cytotoxicity was the shell barrier and controlled release of $Ca(OH)_2$ from these microcapsules (24). PLA and EC are biocompatible biomaterials and are widely used as drug carriers (25, 26). The good biocompatibility of the microcapsules using EC and PLA as shell materials in this study further verified this.

Regarding the prolonged antibacterial effect of microcapsules, it should be noted that the antibacterial effect of these $Ca(OH)_2$ microcapsules on *E. faecalis* lasted longer than pure $Ca(OH)_2$. Compared with formula B, formula A microcapsules prolonged this antibacterial effect. This result was in accordance with the release profile *in vitro* (14). When more EC was used as the shell material, the release of ions was prolonged; therefore, the antibacterial effect was prolonged. However, the CH microcapsules showed a lower antibacterial effect than the positive control on day 1. To evaluate the antibacterial properties of $Ca(OH)_2$ microcapsules, the same amount (weight) of $Ca(OH)_2$ microcapsules as pure $Ca(OH)_2$ was adopted. The drug loading of microcapsules was approximately 80%, which showed that the $Ca(OH)_2$ content of the 2 microcapsules groups was 80% of the positive control group separately. The $Ca(OH)_2$ concentration in the microcapsules was actually lower than that in the positive control. The microcapsule shell delayed the release of ions. These findings may explain the results observed on day 1 when the number of CFUs in the 2 $Ca(OH)_2$ microcapsule groups was higher than in the positive control group. Thus, improving the antibacterial effect in the first action stage by developing multilayer microcapsules should be performed in further studies.

With respect to the markers related to the biomineralization of bone (ie, Col I and OCN), mRNA expression was not detected in the positive control on the first day. The reason that mRNA expression was not detected in the positive control on the first day may be attributed to the cytotoxicity of the positive control group. The up-regulation of Col I and OCN of $Ca(OH)_2$ medicaments was in accordance with previously published studies (23, 27). It was verified that calcium ions from $Ca(OH)_2$ stimulated fibronectin gene expression, and fibronectin could enhance OCN expression (27). Recently, it was reported that calcium ions from $Ca(OH)_2$ up-regulated Col I and OCN expression because of the activation of p38 and JNK (23). Two formulas of $Ca(OH)_2$ microcapsules, especially the formula A group, prolonged the up-regulation of Col I and OCN. This may be attributed to the sustained and controlled-release profile of the microcapsules and indicated that the microcapsules with 80% drug loading and PLA/EC shells might be beneficial to bone mineralization.

$Ca(OH)_2$ was introduced into dentistry by Hermann and occupies a prominent position as a versatile medicament in endodontics. Antimicrobial activity is one of the most important effects of $Ca(OH)_2$. However, the antibacterial effects of $Ca(OH)_2$ were not that perfect in the present study. In order to improve the antibacterial effect, a new reagent with a targeted effect against bacteria is being studied by us, and the effect against bacterial biofilms of this new reagent will be the focus of a further study.

Acknowledgments

The authors deny any conflicts of interest related to this study.

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