Influence of Calcium Hydroxide–loaded Microcapsules on Osteoprotegerin and Receptor Activator of Nuclear Factor Kappa B Ligand Activity

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Abstract

Introduction: Calcium hydroxide ($Ca[OH]_2$) microcapsules were synthesized to allow controlled release of $Ca(OH)_2$. The aim of this study was to evaluate the influence of $Ca(OH)_2$ microcapsules on osteoprotegerin (OPG) activity, receptor activator of nuclear factor kappa B ligand (RANKL) activity, and the OPG/RANKL ratio compared with pure $Ca(OH)_2$ powder and Vitapex (Neo Dental Chemical Products Co Ltd, Tokyo, Japan). **Methods:** One formula of $Ca(OH)_2$ microcapsules was evaluated, and pure $Ca(OH)_2$ powder was used as a control. A commonly used $Ca(OH)_2$ medication containing an oily vehicle (Vitapex) was also evaluated, and the *in vitro* release profile of Vitapex was studied. The human osteosarcoma cell line MG63 was used to evaluate the influence of $Ca(OH)_2$ microcapsules, pure $Ca(OH)_2$ powder, and Vitapex on OPG and RANKL activity. The relative messenger RNA (mRNA) expression of OPG and RANKL was determined by real-time polymerase chain reaction. The protein expression of OPG and RANKL in supernatants was measured using enzyme-linked immunosorbent assay. **Results:** Vitapex prolonged the release of $Ca(OH)_2$ compared with pure $Ca(OH)_2$ powder, and the release rate of Vitapex was faster than that of the microcapsules. The OPG/RANKL ratio in the microcapsules group was up-regulated at both the mRNA and protein levels compared with the negative control group and the pure $Ca(OH)_2$ powder group. The ratio in the Vitapex group was lower than the microcapsule group both at the mRNA and protein levels. **Conclusions:** $Ca(OH)_2$ microcapsules increased the expression of OPG although they did not increase the expression of RANKL compared with pure $Ca(OH)_2$ powder and Vitapex. This increase in expression led to an increase in the OPG/RANKL ratio and eventual inhibition of osteoclast activity. (*J Endod 2014;40:1977–1982*)

Key Words

Calcium hydroxide, microcapsules, osteoclast activity, osteoprotegerin/receptor activator of nuclear factor kappa B ligand, Vitapex

Calcium hydroxide ($Ca[OH]_2$) was introduced into dentistry by Hermann in the 1920s. In endodontics, $Ca(OH)_2$ is mainly used for pulp capping procedures as an intracanal medication, in certain apexification techniques, as a component of several root canal sealers, and in external resorption (1). The induction of hard tissue deposition is an important property and supports the use of $Ca(OH)_2$ as a versatile medication in endodontics (2).

Although $Ca(OH)_2$ can induce hard tissue formation, bone regeneration is a complex phenomenon and is the result of the activities of osteoblasts, osteocytes, and osteoclasts. The interactions between these cells control bone regeneration through the release of cytokines, which control bone differentiation and formation (3).

The development of osteoclasts is controlled by cytokines, such as the receptor activator of nuclear factor kappa B ligand (RANKL) and osteoprotegerin (OPG). The RANKL/receptor activator of nuclear factor kappa B (RANK)/OPG pathway has been shown to be the key regulator of bone remodeling and is directly involved in the differentiation, activation, and survival of osteoclasts and osteoclast precursors. RANKL is mainly produced by osteoblasts under the control of various growth factors, hormones, and cytokines, which activate RANK, found on osteoclasts and preosteoclast precursors. RANK-RANKL interactions lead to preosteoclast recruitment, fusion into multinucleated osteoclasts, osteoclast activation, and osteoclast survival. Osteoblasts produce OPG, which binds to and inactivates RANKL. Therefore, each of these RANK-mediated responses can be fully inhibited by OPG (4).

When $Ca(OH)_2$ contacts the tissue directly, $Ca(OH)_2$ powder is not conducive to local tissue repair because of its high alkalinity and rapid release (5, 6). According to a study by Ferreira et al (7), pure $Ca(OH)_2$ powder can stimulate the production of interleukin (IL)-1β. IL-1β may stimulate osteoclastogenesis by inducing the expression of RANKL (8). This indicates that pure $Ca(OH)_2$ powder may up-regulate the expression of RANKL from osteoblasts and stimulate osteoclast activity.

The ideal vehicle should have the following properties: (1) allow for gradual and slow Ca^{2+} and OH^- ionic release, (2) allow for slow diffusion in the tissues with low solubility in tissue fluids, and (3) exert no adverse effects on the induction of hard tissue deposition. To prolong the release of ions, oily vehicles of $Ca(OH)_2$ medications, such as Vitapex (Neo Dental Chemical Products Co Ltd, Tokyo, Japan),

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were introduced for use in dentistry. However, their cytotoxicity, immunogenicity, low $Ca(OH)_2$ loading, and difficulty of removal limit the application of oily vehicles (2, 9–11). To solve these problems, controlled release $Ca(OH)_2$-loaded microcapsules were developed in a previous study (12). When the microcapsules contact the tissue directly, the shell materials are used as a barrier between $Ca(OH)_2$ and the tissue. These microcapsules prolonged and controlled the release of $Ca(OH)_2$. Further studies verified that the $Ca(OH)_2$ microcapsules reduced cytotoxicity *in vitro* compared with pure $Ca(OH)_2$ powder (13).

In the present study, we evaluated the influence of $Ca(OH)_2$ microcapsules, pure $Ca(OH)_2$ powder (Sinopharm Chemical Reagent Co Ltd, Beijing, China), and a commonly used $Ca(OH)_2$ paste with an oily vehicle (Vitapex) on OPG activity, RANKL activity, and the OPG/RANKL ratio (messenger RNA [mRNA] and protein expression level) expressed by osteoblasts. The microcapsules used in this study were formula A microcapsules, with pure ethylcellulose as the shell material because of its slow release of $Ca(OH)_2$ (12). The *in vitro* release of Vitapex was evaluated to determine its release profile. Pure $Ca(OH)_2$ powder was used as a control. The hypothesis in this study was that $Ca(OH)_2$ microcapsules would increase the ratio of OPG/RANKL both at the mRNA and protein levels compared with pure $Ca(OH)_2$ powder and Vitapex.

Materials and Methods

In the present study, formula A microcapsules with a pure ethylcellulose shell were evaluated. The $Ca(OH)_2$ microcapsules were prepared by the phase separation technique, and the preparation details were described in our previous study (12). In that study, the morphology and composition, particle size distribution, glass transition temperature, drug loading, and encapsulation efficiency were characterized (12). In the study evaluating the *in vitro* release profile, it took 456 hours for the microcapsules to release 90% of the total $Ca(OH)_2$ content (12). The $Ca(OH)_2$ microcapsules exhibited prolonged antibacterial activity and prolonged the up-regulation of bone-related markers with reduced cytotoxicity (13). Vitapex and pure $Ca(OH)_2$ powder were also evaluated in this study.

In Vitro Release Profile

This procedure was the same as that described in our previous study (12). Medications (50 mg) were suspended in 1 mL deionized water and placed in a dialysis bag. The dialysis bag was then placed in a serum bottle containing 100 mL deionized water as a dissolution medium. The serum bottles were maintained at 37°C and shaken at 50 rpm using a THZ-22 constant temperature shaking incubator (Taicang Laboratory Equipment Factory, Taicang, China). Five samples were prepared for each group. At selected times (6, 8, 12, 24, 48, 72, 120, 168, 216, 264, 312, 360, 408, 456, and 504 hours), a 200-μL aliquot was removed, and an equal volume of fresh deionized water was added. The Ca^{2+} concentration in the solution samples was analyzed with a Hitachi 7180 Chemistry Analyzer (Tokyo, Japan) using a calcium kit (Biosino Bio-technology and Science Inc, Beijing, China). The pH of the samples was assessed using a KS701 pH microelectrode (Shindengen Electric Manufacturing Co Ltd, Tokyo, Japan). The release of pure $Ca(OH)_2$ from microcapsules was outlined in our previous study (12), and these results were added to the present study to compare them with those obtained for Vitapex.

Preparation of the Medications and Cells

A Transwell insert (0.4-μm filter; Millipore, Billerica, MA) of a 6-well plate was used to hold the medication. The Transwell insert held 15 mg $Ca(OH)_2$ medication in the $Ca(OH)_2$ microcapsule, Vitapex, and pure Ca(OH)2 powder groups, and 4 mL culture medium was prereleased in the 6-well plate. Each well contained 6 mL culture medium, which was changed every other day. The Transwell inserts that held the prereleased (for 14, 7, 5, 3, 1, and 0 days) $Ca(OH)_2$ medications were used for the experiments. In the negative control, cells were cultured in medium without medicament.

The human osteosarcoma cell line MG63 was purchased from the American Type Culture Collection and cultured as recommended in 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.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 medications were placed in the wells containing cells and cultured for 3 days, respectively, and further experiments detailed later were performed. All experiments were performed in triplicate.

Quantitative Real-time Polymerase Chain Reaction

The cells in the wells were washed twice with phosphate buffered saline and harvested using Trizol reagent (Invitrogen, Carlsbad, CA). RANKL and OPG mRNA expression was detected 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 polymerase chain 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). The sequences of the primers were as follows: OPG forward primer, 5′-GGAACCCCAGAGCGAAATACA-3′; OPG reverse primer, 3′-CCTGAAGAATGCCTCCTCACA-5′; RANKL forward primer, 5′-CAGAAGATGGCACTCACTGCA-3′; RANKL reverse primer, 3′- CAC CATCGCTTTCTCTGCTCT-5′; glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) forward primer, 5′-GAAGGTGAAGGTCGGAGTC-3′; and GAPDH reverse primer, 3′-GAAGATGGTGATGGGATTTC-5′. GAPDH was used as an internal control. 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 using the equation $2^{-\Delta Ct}$ (ΔCt = Ct of target gene minus Ct of GAPDH) (14).

Enzyme-linked Immunosorbent Assay

Before the cells were treated with Trizol reagent, the supernatants in the wells were centrifuged at 1200 rpm for 5 minutes and collected for further analysis. Commercially available human-specific enzyme-linked immunosorbent assay kits were used to quantify RANKL and OPG (R&D, Minneapolis, MN) in these culture supernatants.

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

The *In Vitro* Release of Ca^{2+} and pH Profiles

In vitro release profiles are presented in Figure 1. Figure 1*A* shows that the pH of the surrounding medium in the Vitapex group was greater than 11 at 72 hours, and the pH was maintained at approximately 11.5. In the microcapsules group and the pure $Ca(OH)_2$ powder group, the

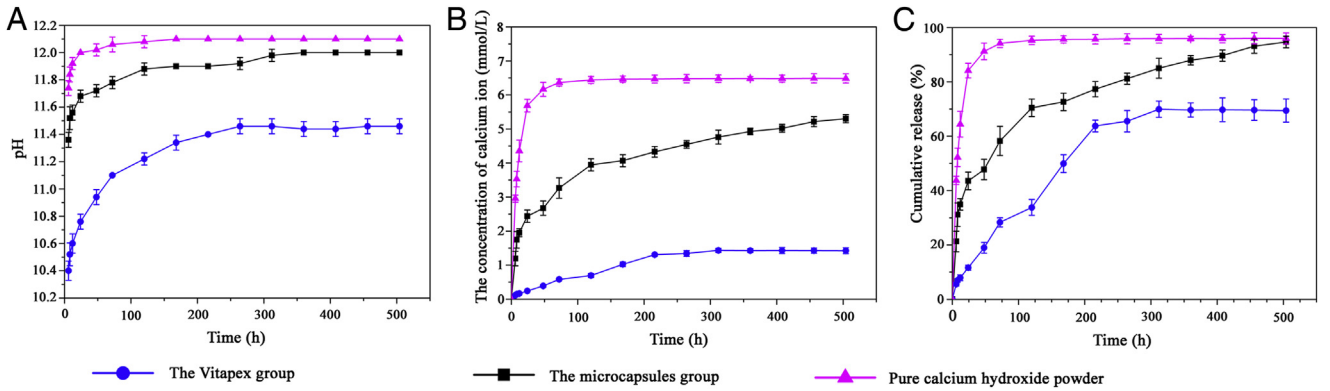

Figure 1. The *in vitro* release profile of the Vitapex group compared with the microcapsules group and the pure $Ca(OH)_2$ powder group. (*A*) The pH profiles of the 3 groups. The pH of the surrounding medium in the Vitapex group was lower than that in the other 2 groups. (*B*) The calcium ion concentration profile in the 3 groups. The Ca^{2+} concentration in the Vitapex group was much lower than that in the other 2 groups. (*C*) The cumulative release rate profile in the 3 groups. The release rate in the Vitapex group was faster than that in the microcapsules group and slower than that in the pure $Ca(OH)_2$ powder group.

pH rapidly reached 11 and was maintained at approximately 12. Figure 1*B* shows that the Ca^{2+} concentration in the surrounding medium in the Vitapex group was consistently lower than 1.5 mmol/L, much lower than that in the other 2 groups. Figure 1*C* shows the cumulative release rate of $Ca(OH)_2$ in the 3 groups. As previously reported, 91% of the total $Ca(OH)_2$ was released within 48 hours in the pure $Ca(OH)_2$ powder group, and it took 456 hours for 90% of $Ca(OH)_2$ to be released in the microcapsules group. In the Vitapex group, the cumulative release rate was stable at approximately 70% at 312 hours and did not increase after this time period.

Influence on OPG, RANKL Activity, and the OPG/RANKL Ratio (mRNA Expression)

The mRNA expression of OPG and RANKL is shown in Figure 2*A* and *B*. Figure 2*C* shows the ratio of OPG/RANKL (mRNA expression). Gene expression in the pure $Ca(OH)_2$ powder group was not detected with the insert prereleased for 0 days. OPG mRNA expression in the microcapsules group was higher than that in the negative control group with inserts prereleased for 0, 3, and 5 days ($P < .05$) and higher than that in the Vitapex group with inserts prereleased for 0, 1, 3, 5, and 7 days ($P < .05$). OPG mRNA expression in the Vitapex group was lower than that in the negative control group with inserts prereleased for 0, 1, 3, 5, and 7 days ($P < .05$). OPG mRNA expression in the pure $Ca(OH)_2$ powder group was higher than that in the negative control group with inserts prereleased for 1 and 3 days ($P < .05$). There was no significant difference in RANKL mRNA expression between the microcapsules group and the negative control group ($P > .05$). RANKL mRNA expression in the Vitapex group was lower than that in the negative control group with inserts prereleased for 1, 3, and 5 days ($P < .05$) and in the microcapsules group with inserts prereleased for 0, 1, 3, and 5 days ($P < .05$). RANKL mRNA expression in the pure $Ca(OH)_2$ powder group was higher than that in the negative control group with inserts prereleased for 1 and 3 days ($P < .05$).

The OPG/RANKL ratio (mRNA expression) in the microcapsules group was higher than that in the negative control group with inserts prereleased for 5 days ($P < .05$) relative to that in the Vitapex group with inserts prereleased for 1, 3, 5, and 7 days ($P < .05$) and the pure $Ca(OH)_2$ powder group with inserts prereleased for 1, 3, 5, and 7 days ($P < .05$). However, both the Vitapex group and the pure $Ca(OH)_2$ powder group decreased the OPG/RANKL ratio (mRNA expression) with inserts prereleased for 1, 3, 5, and 7 days (Vitapex group) and for 1 day (pure $Ca[OH]_2$ powder group) ($P < .05$).

Influence on OPG, RANKL Activity, and the OPG/RANKL Ratio (Protein Expression)

The protein expression of OPG and RANKL is shown in Figure 3*A* and *B*. Figure 3*C* shows the ratio of OPG/RANKL (protein expression). Gene expression in the pure $Ca(OH)_2$ powder group was not detected with the insert prereleased for 0 days. The OPG concentration in the supernatants in the microcapsules group was higher than that in the negative control group with inserts prereleased for 0, 1, 3, and 5 days ($P < .05$) relative to the Vitapex group with inserts prereleased for 1, 3, and 5 days ($P < .05$) and the pure $Ca(OH)_2$ powder group with inserts prereleased for 3 and 5 days ($P < .05$). There was no significant difference in RANKL protein expression between the microcapsules group and the negative control group ($P > .05$). The RANKL concentration in the pure $Ca(OH)_2$ powder group was higher than that in the negative control group ($P < .05$) with inserts prereleased for 1 and 3 days ($P < .05$). The protein expression of RANKL in the Vitapex group was higher than that in the negative control group with inserts prereleased for 7 days ($P < .05$).

The ratio (protein expression) in the microcapsules group was higher than that in the negative control group with inserts prereleased for 1 and 5 days ($P < .05$) relative to the Vitapex group with inserts prereleased for 1, 3, 5, and 7 days ($P < .05$) and the pure $Ca(OH)_2$ powder group with inserts prereleased for 3 and 5 days ($P < .05$).

Discussion

In the present study, the hypothesis that $Ca(OH)_2$ microcapsules would increase the expression of OPG without increasing the expression of RANKL and then increase the ratio of OPG/RANKL *in vitro* compared with pure $Ca(OH)_2$ powder and Vitapex both at the mRNA and protein levels was confirmed.

In certain dental conditions, such as external resorption (1), sustained release of $Ca(OH)_2$ is required. Oily vehicles are often used to slow the release of $Ca(OH)_2$; however, oily vehicles cannot achieve high $Ca(OH)_2$ loadings such as those possible with aqueous vehicles. Vitapex is composed of $Ca(OH)_2$ (30.3%), iodoform (40.4%), silicone oil (22.4%), and other undescribed substances (6.9%) (2). Vitapex is a classic $Ca(OH)_2$ medication with an oily vehicle, and its drug loading is only 30.3%. Because of low $Ca(OH)_2$ loading, the concentration of Ca^{2+} and the pH in the *in vitro* profile were lower than those in the other 2 groups. The drug loading of $Ca(OH)_2$ microcapsules was approximately 80%. Therefore, the $Ca(OH)_2$ content in the microcapsules group was

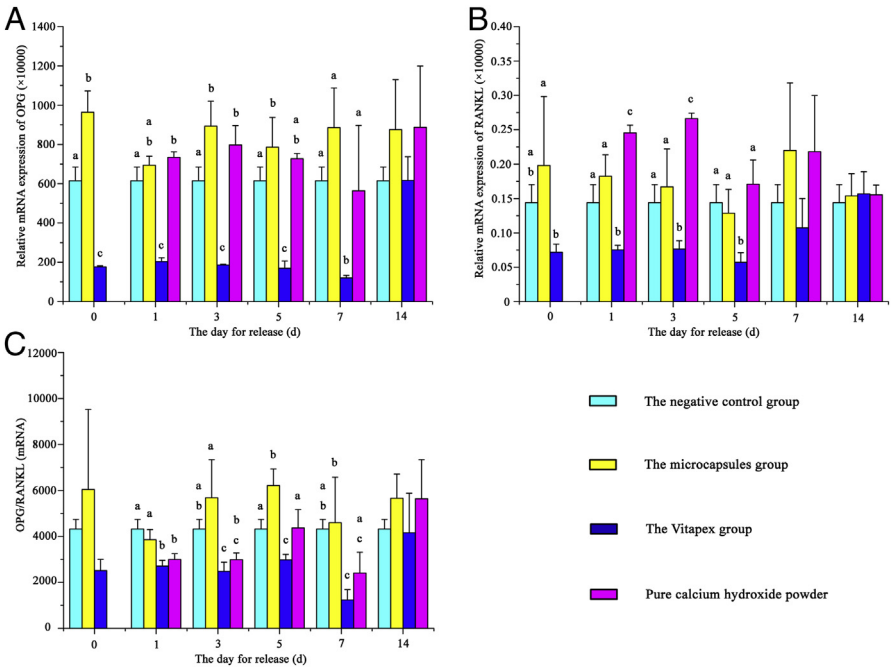

Figure 2. The relative mRNA expression in the 4 groups with inserts prereleased for various time periods. The results represent the mean standard ± deviation. Different superscript letters indicate statistical differences between the groups ($P < .05$). Gene expression in the pure $Ca(OH)_2$ powder group was not detected with inserts prereleased for 0 days. (*A*) The relative mRNA expression of OPG with inserts prereleased for various time periods. The mRNA expression in the microcapsules group was up-regulated with inserts prereleased for 0, 3, and 5 days ($P < .05$). The mRNA expression in the Vitapex group was down-regulated with inserts prereleased for 0, 1, 3, 5, and 7 days ($P < .05$). The mRNA expression in the pure $Ca(OH)_2$ powder group was up-regulated with inserts prereleased for 1 and 3 days ($P < .05$). (*B*) The relative mRNA expression of RANKL with inserts prereleased for various time periods. RANKL mRNA expression in the microcapsules group was not up-regulated ($P > .05$). RANKL mRNA expression in the Vitapex group was down-regulated with inserts prereleased for 1, 3, and 5 days ($P < .05$). RANKL mRNA expression in the pure $Ca(OH)_2$ powder group was up-regulated with inserts prereleased for 1 and 3 days ($P < .05$). (*C*) The ratio of OPG/RANKL (mRNA) with inserts prereleased for various time periods. The ratio in the microcapsules group (mRNA expression) was up-regulated with inserts prereleased for 5 days ($P < .05$). The ratio in the Vitapex group (mRNA expression) was down-regulated with inserts prereleased for 1, 3, 5, and 7 days ($P < .05$). The ratio in the pure $Ca(OH)_2$ powder group (mRNA expression) was down-regulated with inserts prereleased for 1 day ($P < .05$).

higher than that in Vitapex with the same weight of medication. Although Vitapex prolonged $Ca(OH)_2$ release relative to pure $Ca(OH)_2$ powder group, its release rate was faster than the microcapsules group. This showed that microcapsulation may be a better method of prolonging the release rate of medications.

Biocompatible microparticles and nanoparticles designed for controlled drug delivery have been widely investigated and successfully applied in the medical field (15–17). These new drug delivery systems have the advantages of controlled release of the drug, targeted drug delivery, reduced side effects, and improved therapeutic effects. These novel drug release systems were also recently introduced into the fields of endodontics and periodontics (12, 16, 18–22). $Ca(OH)_2$ microcapsules can result in the sustained release of $Ca(OH)_2$. The core-shell heterostructure of microcapsules was verified in a previous study using transmission electron microscope (TEM) (12). The biocompatible polymer shell materials could act as a protective barrier between $Ca(OH)_2$ and tissue. Based on the previously described reasons, the $Ca(OH)_2$ microcapsules not only reduced the cytotoxicity but also up-regulated the expression of OPG, reducing the up-regulation of RANKL because of pure $Ca(OH)_2$ powder. These factors could lead to reduced osteoclast activity and an increase in osteogenic activity.

The human osteoblast cell line (MG63) with the capacity for remodeling was used in this study to avoid the heterogeneity of osteoblasts or odontoblasts derived from primary culture whose function might be influenced by the cell source and cell generation. The MG63 osteoblast-like cells express high levels of OPG and inducible RANKL and can be used as an experimental model to assess the ability of materials to modify the expression of these 2 molecules that play a key role in the pathophysiology of bone remodeling (23).

OPG and RANKL were not detected in the pure $Ca(OH)_2$ powder group with inserts prereleased for 0 days. According to a previous study, the pure $Ca(OH)_2$ powder group showed cytotoxicity when the $Ca(OH)_2$ concentration was 1000 μg/mL (13). In the present study, the actual $Ca(OH)_2$ concentration of the pure $Ca(OH)_2$ powder group with inserts prereleased for 0 days was 1500 μg/mL and may have been more toxic to the cells.

The RANKL-RANK-OPG system is 1 of the principal mediators in the maintenance of bone cell function and the activation of bone remodeling. Because the interaction between RANKL and RANK depends on the balance between RANKL and OPG, the OPG/RANKL ratio is the main indicator and plays a crucial role in orientating the pathophysiological evolution of bone remodeling and decides the balance between bone formation and resorption (24). We observed that the OPG/RANKL ratio was not increased in the pure $Ca(OH)_2$ powder group. The ratio in the pure $Ca(OH)_2$ powder group even decreased compared with the negative control group during the study. The reason for this may be the up-regulation of IL-1β and tumor necrosis factor alpha induced by pure $Ca(OH)_2$ powder (7, 25). This would result in the up-regulation of RANKL (8). In contrast, the OPG/RANKL ratio was increased in the microcapsules group. These results showed that the $Ca(OH)_2$ microcapsules prohibited the differentiation, activation, and survival of osteoclasts and osteoclast precursors.

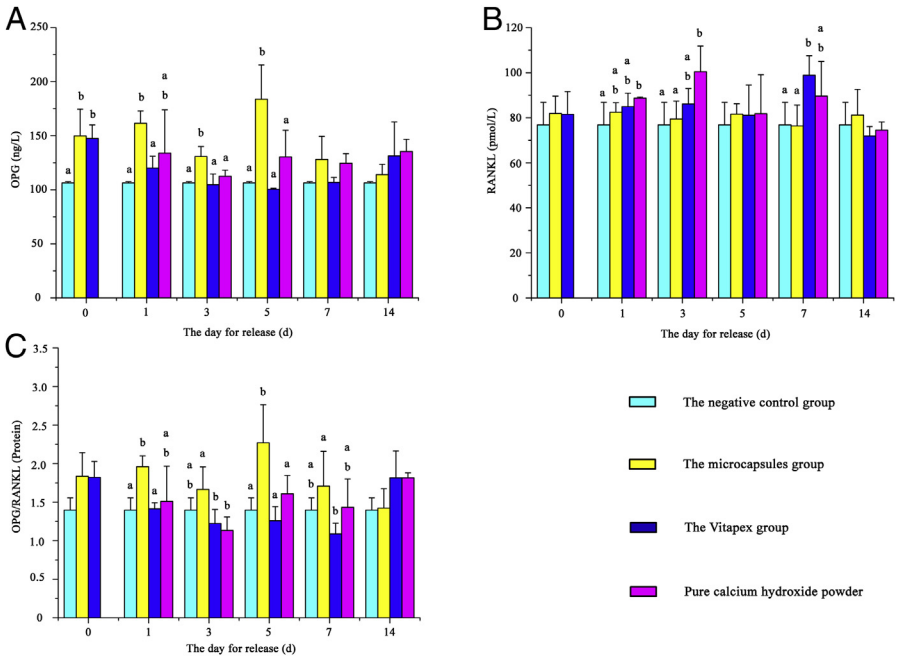

Figure 3. Protein expression in the 4 groups with inserts prereleased for various time periods. The results represent the mean ± standard deviation. Different superscript letters indicate statistical differences between the groups ($P < .05$). Gene expression in the pure $Ca(OH)_2$ powder group was not detected with inserts prereleased for 0 days. (*A*) The protein expression of OPG with inserts released for various time periods. OPG protein expression in the microcapsules group was up-regulated with inserts prereleased for 0, 1, 3, and 5 days ($P < .05$). OPG protein expression in the Vitapex group was up-regulated with inserts prereleased for 0 days ($P < .05$). (*B*) The protein expression of RANKL with inserts prereleased for various time periods. RANKL protein expression in the microcapsules group was not up-regulated ($P > .05$). RANKL protein expression in the Vitapex group was up-regulated with inserts prereleased for 7 days ($P < .05$). RANKL protein expression in the pure $Ca(OH)_2$ powder group was up-regulated with inserts prereleased for 1 and 3 days ($P < .05$). (*C*) The ratio of OPG/RANKL (protein) with inserts prereleased for various time periods. The ratio in the microcapsules group (protein expression) was up-regulated with inserts prereleased for 1 and 5 days ($P < .05$).

The OPG/RANKL ratio in the Vitapex group was lower than that in the microcapsules group. These results may have been caused by the following 2 reasons: the lower $Ca(OH)_2$ loading of Vitapex would not increase the expression of OPG compared with the microcapsules group, and $Ca(OH)_2$ is only 1 of many ingredients contained in Vitapex; it is speculated that silicone oil, iodoform, and other components would have an adverse impact.

$Ca(OH)_2$ microcapsules should be placed in the canal in patients undergoing pulpotomy and apexification and in those with external resorption. This *in vitro* study was performed over14 days because there were no statistical differences between the groups at 14 days. Because of the large release contact area and constant liquid change, the experimental conditions in this *in vitro* study were different from the conditions *in vivo*. The diffusion rate of $Ca(OH)_2$ was faster than that in the *in vivo* condition. In order to achieve a deeper understanding of the effects of $Ca(OH)_2$ microcapsules *in vivo*, it is important to evaluate the long-term effect of $Ca(OH)_2$ microcapsules in an animal model in future studies.

In conclusion, we suggest that $Ca(OH)_2$ microcapsules may act on osteoblastlike cells to alter the OPG/RANKL ratio such that osteoclastogenesis is impaired. Thus, an increase in the OPG/RANKL ratio would promote bone healing. Pure $Ca(OH)_2$ powder and Vitapex did not increase this ratio.

Acknowledgments

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

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