Effects of iRoot SP on Mineralization-related Genes Expression in MG63 Cells

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Abstract

Introduction: The purpose of this study was to assess the effects of iRoot SP root canal sealer (Innovative BioCreamix Inc, Vancouver, Canada) on the expression of mineralization-related genes in human MG63 osteoblast-like cells. **Methods:** Specimens (5 mm in diameter and 2 mm in height) of iRoot SP and AH Plus (Dentsply DeTrey, Konstanz, Germany) were extracted from a 5-mL culture medium. The MG63 cells were exposed to various dilutions (1/1, 1/2, and 1/4) of the extracts. The 3,(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide MTT assay was used for assessing the dental materials' nonspecific cytotoxicity. The expression of mineralization-related genes, including collagen type I (COL I), osteocalcin (OCN), bone sialoprotein (BSP) and osteopontin (OPN), was detected on days 1, 3, and 6 by a real-time polymerase chain reaction. An enzyme-linked immunosorbent assay experiment was used for evaluating COL I and BSP protein changes. The data were analyzed with one-way analysis of variance and Tukey tests. **Results:** In the MTT assay, the undiluted extracts of iRoot SP were noncytotoxic, whereas the undiluted extracts of AH Plus were rated as slightly cytotoxic. iRoot SP up-regulated COL I, OCN, and BSP messenger RNA expression after 3 and 6 days. In the enzyme-linked immunosorbent assay experiment, iRoot SP increased COL I and BSP protein levels compared with AH Plus and the control group on day 6. **Conclusions:** In the presence of iRoot SP, MG63 cells can produce more mineralized matrix gene and protein expression. Based on these results, iRoot SP can be considered as a favorable material for cell-material interaction. *(J Endod 2010;36:1978–1982)*

Key Words

Cytoxicity, enzyme-linked immunosorbent assay, mineralization, real-time polymerase chain reaction, root canal sealer

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The ideal root canal filling material must possess certain characteristics, including biocompatibility, adequate marginal sealing quality, dimensional stability, and the ability to allow or induce bone repair and antimicrobial activity (1). Biocompatibility is one of the factors that influence the clinician's choice of filling materials in root canal treatment as the materials might be placed in contact with periapical tissues (2). Furthermore, the tissues' responses to these materials might influence the outcome of the root canal treatment (3). Many endodontic sealers are now used in clinical practice, including zinc oxide–eugenol cements, glass ionomer cements, epoxy resins, and Portland-based cements, but none meets all the appropriate requirements.

iRoot SP (Innovative BioCreamix Inc, Vancouver, Canada), a new root canal sealer, is a convenient, premixed, ready-to-use injectable white hydraulic cement paste developed for permanent root canal filling and sealing applications. According to the manufacturer, iRoot SP is an aluminum-free, hydrophilic, calcium silicate–based material that requires the presence of water to set and harden. iRoot SP requires no additional curing agent or mixing, and it delivers a consistent, homogeneous product for filling root canals with or without gutta-percha points (4). iRoot SP has a composition similar to white mineral trioxide aggregate and has excellent physical properties and antimicrobial activity (5). Superior qualities and handling abilities make iRoot SP an innovative and novel endodontic material as a root canal sealer (4, 5). Although the sealing ability and antimicrobial activity of iRoot SP have been investigated, the biocompatibility and effects of iRoot SP on messenger RNA expression of several mineralization-related genes are still not clear (4, 5). AH Plus (Dentsply DeTrey, Konstanz, Germany) is a popular resin-based sealer used for permanent root canal sealing. This sealer provides outstanding long-term dimensional stability and presents optimal handling and working time (6).

The purpose of the present study was to evaluate iRoot SP's biocompatibility and effects on the expression of mineralization-related genes (type I collagen, osteocalcin, bone sialoprotein, and osteopontin) during hard-tissue formation in osteoblast-like MG63 cells while also comparing the results with those of AH Plus root canal sealer.

Materials and Methods

Material Preparation

In this experiment, iRoot SP and AH Plus were used. Material specimens were prepared (5 mm in diameter and 2 mm in height, $n = 6$ per material) in sterile plastic molds following the manufacturers' directions under sterile conditions. The specimens were allowed to set in a humidified 5% CO_2, 95% air atmosphere for 24 hours at 37°C. Then each test specimen was eluted in 5 mL of Dulbecco's modified Eagle medium (DMEM) (Gibco, Grand Island, NY) at 37°C in a humidified 5% CO_2, 95% air atmosphere for 24 hours. The medium was drawn off and filtered sterile at 0.22 μm. Subsequently, various dilutions (final dilution: 1/1, 1/2, and 1/4) of these extraction media were prepared for use in this study. The pH of the extracts was checked using a twin pH meter (Merck, Darmstadt, Germany).

Cell Culture

MG63 cells have been a commonly used model for human osteoblasts and widely used for testing biomaterials. The cells were cultivated in DMEM supplemented with 10% fetal bovine serum (FBS) (Gibco), 50 U/mL of penicillin, and 50 μg/mL of streptomycin (Gibco) at 37°C in an air atmosphere containing 5% CO2 and 95% relative humidity. Cells at a logarithmic growth phase were detached with 0.5 g/L of trypsin

and 0.2 g/L of ethylene diamine tetraacetic acid (Hyclone, Logan, UT) in phosphate-buffered saline solution and used for cell inoculation. Cells were subcultured twice per week, and the culture was maintained at a passage number of 3 to 7. For the mineralization assay, 50 μg/mL of ascorbic acid (Sigma-Aldrich, St Louis, MO), 10 mmol/L of β-glycerophosphate (Sigma-Aldrich), and test specimen extracts were added to the culture medium. The culture medium without any extracts of root canal sealer was the control.

3,(4,5-dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide Assay

The 3,(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was conducted according to Guigand et al (7). Cells were seeded in a 96-well plate (Invitrogen, Carlsbad, CA) at the density of 1,000 cells per well suspended in 100 μL of DMEM. After incubation for 24 hours, the medium was aspirated from all wells and replaced with 100 μL/well of extract medium of three materials (final dilution: 1/1, 1/2, and 1/4) or a control medium and incubated for another 24 hours. Next, succinate dehydrogenase activity was then determined by adding 20 μL of a 5 mg/mL of MTT (Invitrogen, Carlsbad, CA) to each well followed by 4 hours of incubation. After incubation, the MTT and medium were aspirated, and the formazan product was solubilized in 100 μL of 0.04 mol/L HCl in isopropanol. The spectrophotometric absorbance at 570 nm was then measured using a FLUOstar Optima (BMG Labtech, Offenburg, Germany). Six replicates of each extract or control were performed in each test. All assays were repeated at least twice to ensure reproducibility. The absorption value obtained with the control was deemed to indicate 100% viability. Cytotoxicity responses were rated as severe (<30%), moderate (30%-60%), slight (60%-90%), or noncytotoxic (>90%) based on the activity relative to values obtained for the controls (8).

Mineralization Assay

The MTT assay indicated that AH Plus undiluted extracts were rated slightly cytotoxic so undiluted extracts were excluded from the mineralization experiments. MG63 cells were cultured in six-well plates at the density of 1×10^5 cells per well. After 24 hours, the cells were switched to DMEM containing 5% FBS, mineralization media (50 μg/mL ascorbic acid and 10 mmol/L of β-glycerophosphate), and extracts of the test specimen in 1/2 and 1/4 dilution. Mineralization of extracellular matrix was determined on day 14 by alizarin red S staining (Sigma, St Louis, MO) (9). Briefly, the cells were rinsed twice with phosphate buffered solution (PBS) followed by fixation with 75% ethanol for 10 minutes at room temperature. The cells were then stained with 40 mm of alizarin red S (pH = 4.2) for 10 minutes at room temperature and extensively rinsed with deionized water for 15 minutes. The mineralization experiments were repeated two times (triplicate for each extract).

Total RNA Preparation and Real-Time Polymerase Chain Reaction

The material extracts (final dilution: 1/4) were prepared for use in this study. The MG63 cells were exposed to the elution of iRoot SP, AH Plus, and culture medium for 1, 3, and 6 days. Total RNA was extracted from cells cultured in six-well plates by using TRIzol reagent (Gibco) as instructed by the manufacturer. The RNA was dissolved in Rnase-free water and stored at −80°C until used for reverse-transcriptase polymerase chain reaction (PCR). Complementary DNA was synthesized from the total RNA with reverse transcriptase and oligo(dT)20 primers in a first-strand complementary DNA synthesis kit (Toyobo, Osaka, Japan).

The real-time PCR was conducted using specific primes for type I collage (COL I), osteocalcin (OCN), bone sialoprotein (BSP), and osteopontin (OPN) with SYBR green real-time PCR kit (Toyobo). Real-time PCR was performed on a volume of 20 μL containing 2 μL of complementary DNA, 2 μL of Plus solution, 10 μL of mix-plus (SYBR Green PCR Master Mix-Plus, Toyobo), 0.8 μL of each primer (10 pmol/L), and 4.4 μL of diethyl pyrocarbonate-treated water (Toyobo) using an ABI PRISM 7500 Sequence Detection System Thermal Cycler (Applied Biosystems, Foster City, CA). The specific gene primers for each gene are shown in Table 1. The program was set at 95°C for 60 seconds followed by 45 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 15 seconds, and extension at 72°C for 45 seconds. The reaction product was quantified by Sequence Detection Software V1.3.1 (Applied Biosystems) with β-actin as the reference gene. Melting curve analysis was also performed after the PCR amplification to confirm the absence of the primer dimer in the PCR products. The data are expressed as relative quantity, and the differences are shown as the expression ratio of the normalized target gene according to the software results. Each sample was tested in triplicate.

Enzyme-Linked Immunosorbent Assay

The MG63 cells were cultured in DMEM containing 5% FBS, mineralization media, and extracts of test specimen in 1/4 dilution for 3 and 6 days. The conditioned medium was collected and stored at −80°C until use. Levels of COL I and BSP in the culture supernatant were determined by a human COL I and BSP enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. COL I and BSP concentrations were calculated from a calibration curve by using human COL I and BSP as the standard.

Statistical Analysis

Statistical analysis was performed by one-way analysis of variance followed by a Tukey test. The criterion for statistical significance was defined as $p < 0.05$ using the SPSS13.0 for Windows (SPSS Inc, Chicago, IL) statistical package.

TABLE 1. Quantitative Real-time PCR Primer Information

Target Gene	Primer Sequence (5′ to 3′)	Product Size
COL I	Forward: 5′-TCTGGCGCTCCCATGGCTCT -3′ Reverse: 5′-GCCCTGCGGCACAAGGGATT -3′	166 bp
OCN	Forward: 5′-AGGTGCAGCCTTTGTGTCCA-3′ Reverse: 5′-TGTGGTCAGCCAACTCGTCA-3′	164 bp
BSP	Forward: 5′-ACGGCACCAGTACCAACAGCA-3′ Reverse: 5′-GGTGCCCTTGCCCTGCCTTC-3′	143 bp
OPN	Forward: 5′-AGGCATCACCTGTGCCATACCA -3′ Reverse: 5′-ACTTGGAAGGGTCTGTGGGGCT -3′	154 bp
β-actin	Forward: 5′-GAGCCTCGCCTTTGCCGATCC -3′ Reverse:5′-GCCCCACGATGGAGGGGAAGA -3′	161 bp

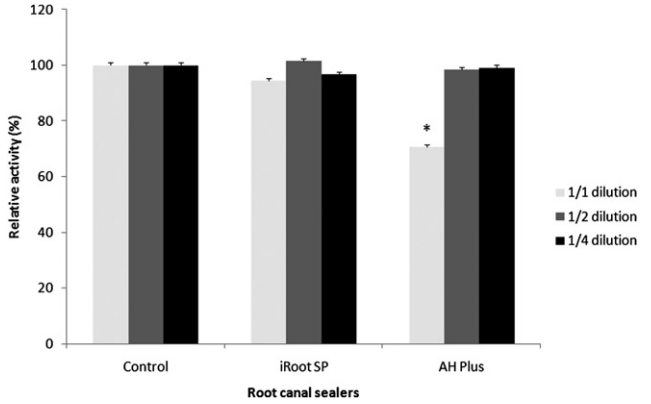

Figure 1. MTT-reducing activity relative to the control of MG63 cells after 24 hours exposure to extracts (1/1, 1/2, and 1/4 dilution) made from 24-hour test specimens. Data are mean ± standard deviation ($n = 6$). *Denotes significant differences from control values with $p < 0.05$.

Results

MTT Assay

In a set state after 24 hours, undiluted extracts of iRoot SP were noncytotoxic, whereas AH Plus undiluted extracts were rated slightly cytotoxic. Extracts of iRoot SP and AH Plus were noncytotoxic in 1/2 and 1/4 dilution (Fig. 1).

Mineralization of MG63 Cells

Calcified nodule formation was determined by alizarin red S staining. The results showed that the mineralization of MG63 cells was increased significantly by iRoot SP in 1/4 dilution on day 14 (Fig. 2).

Gene Expression Results

COL I. On day 1, there was decreased expression in the iRoot SP-treated group compared with the control group ($p < 0.05$). On day 3, the iRoot SP-treated group had a higher Col I expression than that of the AH Plus-treated and control group ($p < 0.05$). On day 6, there were no differences among the groups (Fig. 3).

OCN. There was no expression in three groups on day 1. The iRoot SP-treated group exhibited a higher osteocalcin expression than those of the AH Plus-treated and control group on days 3 and 6 ($p < 0.05$) (Fig. 3).

BSP. Decreased expression was noted in the iRoot SP-treated group compared with the control group on day 1 ($p < 0.05$). No differences were observed among the groups on day 3. BSP expression was slightly increased in the iRoot SP-treated group in comparison with the AH Plus-treated and control group on day 6 ($p < 0.05$) (Fig. 3).

OPN. There was no OPN expression on day 1 and no differences among the groups on days 3 and 6 (Fig. 3).

ELISA for the Detection of COL I and BSP

To determine whether changes in COL I and BSP protein levels paralleled changes in gene expression, we collected conditioned medium and examined them by ELISA. In basic agreement with the observed effects on COL I and BSP messenger RNA levels, iRoot SP increased COL I and BSP protein levels compared with AH Plus and the control group on day 6 ($p < 0.05$) (Fig. 4).

Discussion

A wide variety of root canal sealers are commercially available. The biological compatibility of sealers is essential because during root filling a small amount of cement might affect periradicular tissues either by direct contact, as in cases of overfilling, or by leached components that are released into the surrounding tissues through dentinal tubules, accessory or lateral canals, and apical foramina (10). Therefore, an extract of the test material was used in this study. The extract offers the advantage of being easily sterilized by filtration, and it has the ability to be examined for the effect of materials on cells. The use of extract also simulates the clinical situation in which toxic elements of the materials leach into the periapical tissue.

The MTT assay was used because it is a well-established method for dental material testing (11). The results of the current study showed iRoot SP exhibited good biocompatibility with the MG63 cells. The explanation might be that iRoot SP is a cement consisting of fine hydrophilic particles and aluminum-free calcium silicate composition. The principle compounds in iRoot SP are calcium phosphate, calcium silicates, zirconium oxide, and calcium hydroxide, some of which are

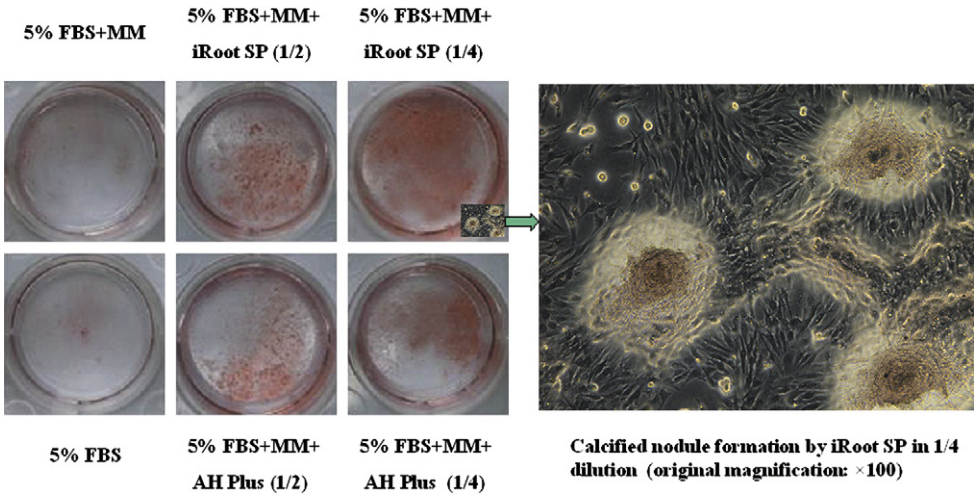

Figure 2. Calcified nodule formation and alizarin red S staining on day 14. The mineralization of the MG63 cells was increased significantly by iRoot SP in 1/4 dilution. (This figure is available in color online at www.aae.org/joe/.)

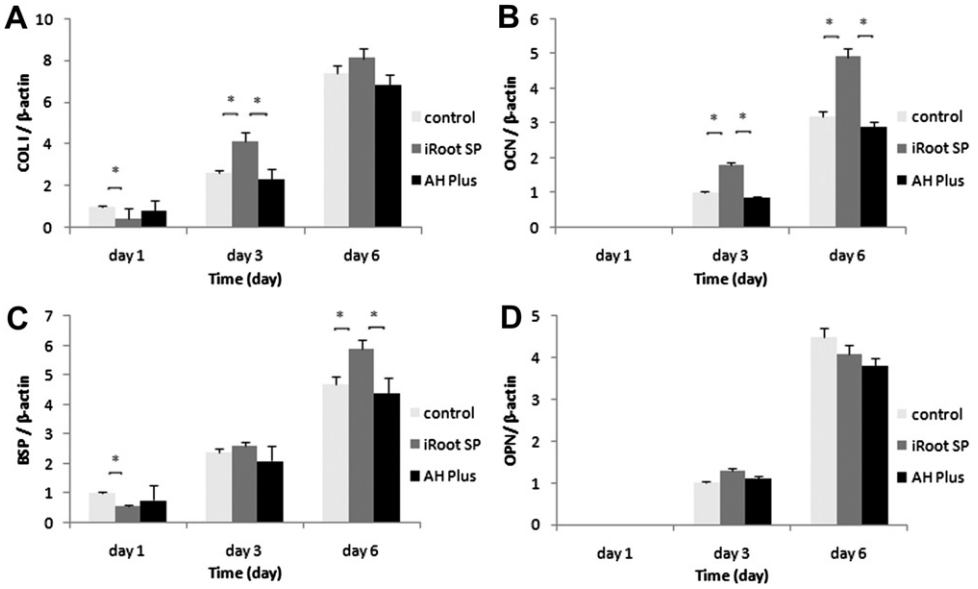

Figure 3. The relative quantity of gene expression normalized to housekeeping gene β-actin on days 1, 3, and 6. (*A*) COL 1, (*B*) OCN, (*C*) BSP, and (*D*) OPN. $^*p < 0.05$.

similar to dental hard tissues. Moisture is supposed to facilitate the hydration reactions of calcium silicates to produce calcium silicate hydrogel (C-S-H) and calcium hydroxide (CH) (5). Calcium hydroxide partially reacts with the phosphate to form hydroxyapatite and water. The water is supposed to start the reaction cycle again and react with calcium silicates to produce calcium silicate hydrogel and calcium hydroxide (12). The high levels of calcium leached out from CH and C-S-H might account for the cement's biocompatibility (13).

AH Plus was selected because of its widespread use. The AH Plus specimen showed a slight cytotoxic effect after 24 hours in the MTT assay. The current study showed that AH Plus displayed a higher cytotoxicity than iRoot SP. This might have been caused by minute amounts of formaldehyde from the sealer or by the release of the sealer's amine and epoxy resin components (14, 15). Overall, the results obtained in this study largely confirmed those in previous reports (16, 17).

COL I, OCN, BSP, and OPN take part in forming a mineralized matrix. COL I expression is an essential component of the extracellular matrix, constituting approximately 90% of the total organic matrix in mature bone (18). Together with mineral, collagen governs the mechanical properties and functional integrity of the osseous tissue. From a number of *in vitro* studies, COL I had been proposed to induce osteoblast differentiation and facilitate mineralization (19). In our study, type I collagen expression was evident from the first day of culture, and the results show a time-dependent increased expression in three groups. In addition, type I collagen expression by MG63 increased in the iRoot SP-treated group compared with the AH Plus and the control group on day 3. OCN is a major noncollagenous protein found in bone and dentin that plays a role in regulating mineralization and calcium ion homeostasis (20). In the present study, the iRoot SP-treated group had a higher OCN expression than those of the AH Plus–treated and control group on days 3 and 6, and expression increased as the culture time increased. Our findings agree with those in previous reports on the production of OCN by osteoblastic cells (20, 21). The expression of BSP is highly specific for mineralizing tissues, including bone, mineralizing cartilage, dentin, and cementum. In mineralized bone matrix, the highest BSP concentration is found in areas in which bone is newly synthesized or remodeled (22, 23). In our studies, BSP expression by the MG63 cells after day 1 was evident. The iRoot SP group showed a higher level of BSP expression than those of the AH Plus and control group on day 6. OPN is an extracellular structural protein and is related both to bone formation and to resorption, and it is also reported that OPN is related to host defenses or tissue repair (24). In this study, OPN expression was evident from day 3 in the groups, and no differences were seen among the groups.

In the ELISA experiment, we observed that iRoot SP increased COL I and BSP proteins expressions in the 1/4 dilution on day 6. The increased expression of the mineralization gene, COL I, and BSP protein in MG63 cells suggests that the mineralization process can proceed by

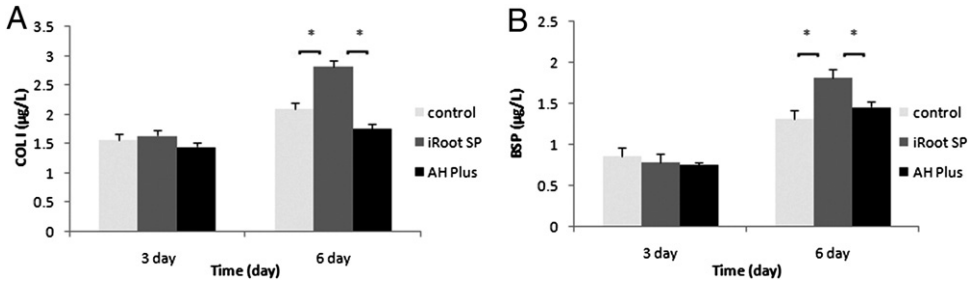

Figure 4. ELISA assay for the detection of COL I and BSP on days 3 and 6. $^*p < 0.05$.

the treatment of iRoot SP under experimental conditions. In addition, iRoot SP can promote mineralization and the early stages of bone matrix formation. The definite mechanism by which iRoot SP influenced mineralization-related gene expression was not well understood. We hypothesize that iRoot SP has favorable properties with regard to the biologic responses of the cells and influences cellular differentiation. In the hydrated iRoot SP, the main reaction products were calcium silicate hydrate and calcium hydroxide. Silicate might be released and be generally a stable substance (25). Calcium hydroxide dissociates into Ca^{2+} and OH^- in an aqueous solution. It has been shown that OH^- and other alkaline substances did not induce mineralization *in vivo* (26). Nevertheless, calcium hydroxide partially reacts with the phosphate to form hydroxyapatite. It was reported that osteoblast differentiation was enhanced by Ca^{2+} released from HA through L-type calcium channel and CaMK2 that regulated the production of OPN and BSP (27). Therefore, hydroxyapatite probably contributed to increased mineralization gene expression and acted as a calcification activator (27–29). Previous reports and the present observations imply that calcium ions released from the root canal sealer might induce the mineralization activity of osteoblast cells (30). Further studies are required to clarify the bioactive components be released from the iRoot SP material.

In this study, the pH of the extract of iRoot SP was stable within the range from 7.5 to 7.8 throughout the experimental time period. The explanation might be that iRoot SP contains a calcium hydroxide component and produces calcium hydroxide when in contact with moisture. Although the released-ionic products of iRoot SP dissolution create a favorable environment for MG63 cells' mineralization *in vivo*, a rapid pH shift of the culture medium could be unfavorable for cellular metabolism (26, 31). The higher pH may have an influence on gene expression in the early stages. Therefore, iRoot SP was found to slightly decrease COL I and BSP expression on day 1.

On the basis of the results of this study, iRoot SP not only induced expression of mineralized-tissue-associated markers, but it also regulated the messenger RNA expression and mineralization of the MG63 cells. It can be stated that iRoot SP has favorable properties regarding the biologic response of the MG63 cells. iRoot SP might be a promising root canal sealer for the healing of periapical tissues clinically.

Conclusions

Our research shows that iRoot SP is not cytotoxic to the MG63 cells. In addition, iRoot SP allows the expression of matrix genes involved in mineralization. It suggests that iRoot SP is a promising root canal sealer for widespread use.

Acknowledgments

The authors deny any conflict of interest.

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