Simvastatin Promotes Dental Pulp Stem Cell–induced Coronal Pulp Regeneration in Pulpotomized Teeth

Weiqian Jia, MD, Yuming Zhao, PhD, Jie Yang, MD, Wenjun Wang, PhD, Xu Wang, MD, Long Ling, MD, and Lihong Ge, PhD

Abstract

Introduction: Pulpotomy is a specific treatment used to save root pulp, in which only the inflamed coronal pulp is removed and capping materials are placed. Our study aims to study the effect of simvastatin (1) on the proliferation and differentiation of dental pulp stem cells (DPSCs) and (2) on DPSC-induced pulp regeneration after pulpotomy. Methods: DPSCs were treated with different concentrations of simvastatin. Cell counting kit-8 activity was examined to test cell proliferation, and alkaline phosphatase assays and alizarin red S staining were conducted to examine differentiation. In addition, DPSCs pretreated with simvastatin were transplanted into the dorsum of CB-17 severe combined immunodeficiency mice. Areas of mineralized tissue were compared. Eighteen immature premolars from 2 beagle dogs were divided into 4 groups and treated by pulpotomy: the mineral trioxide aggregate, absorbable gelatin sponge, cDPSCs, and simvastatin groups. The teeth were extracted after 10 weeks, and the areas of regenerated pulp and dentin were calculated and compared. Results: Simvastatin at 1 μmol/L suppressed cDPSCs proliferation but significantly increased alkaline phosphatase activity and mineral nodule formation. In addition, cDPSCs pretreated with 1 μmol/L simvastatin formed significantly more mineralized tissue in CB-17 severe combined immunodeficiency mice. In the in vivo study, the ratios of the areas of pulp and dentin regeneration were 47.3% ± 2.5%, 76.8% ± 4.3%, and 85.8% ± 0.9%, respectively, in the absorbable gelatin sponge, cDPSCs, and simvastatin groups. Conclusions: Simvastatin stimulates cDPSCs mineralization both in vivo and in vitro. It also promotes DPSC-induced pulp and dentin regeneration after pulpotomy. (J Endod 2016;42:1049–1054)

Key Words
Beagle dog model, dentin regeneration, pulpotomy, simvastatin, stem cell differentiation

Significance
This research explores that simvastatin stimulates DPSCs mineralization, thus can be a promising biocompatible pulp-capping material that has the potential to induce pulp regeneration and de novo dentin formation. Pulpotomy is a vital therapy modality that makes pulp regeneration possible if conducted using stem cells therapy with proper capping material. With tissue engineering attractive both in laboratorial and clinical study, dentin and pulp regeneration may be realized in the near future.

Dentin and pulp are susceptible to damage by caries and trauma. Initial pulpal damage often results in pulpitis, which is commonly symptomatic. As pulpal injury progresses, it may result in liquefaction necrosis of the dental pulp. In patients with immature teeth, pulpal necrosis arrests root development and increases susceptibility to fractures. Pulpotomy is a vital therapy modality that promotes continued root development if conducted before total pulpal necrosis.

Stem cell–based tissue engineering has recently been identified as a promising alternative for dentin regeneration. To further restore tooth integrity, stem cells can be implanted in the pulp chamber, whereby the coronal pulp can be maintained and dentin regeneration achieved.

Human dental pulp has a subpopulation of cells with the phenotypic characteristics of stem cells, as indicated by their strong proliferative and self-renewal potentials, multilineage differentiation, and expression of multiple mesenchymal stem cell surface markers (1). Simvastatin (SIM), an inhibitor of the competitive 3-hydroxy-3-methylglutaryl coenzyme A reductase, is a convenient and economical drug widely used to treat hyperlipidemia. Previous studies have shown that SIM stimulates the expression of bone morphogenetic protein (BMP)-2 (2, 3), thereby promoting bone formation by bone marrow stem cells. Similarly, it has been also shown to stimulate the mineralizing phenotype in dental pulp stem cells (DPSCs). Okamoto et al (4) reported that SIM-treated DPSCs showed enhanced odontogenic differentiation and accelerated mineralized tissue formation. Furthermore, Min et al (5) and Karanxha et al (6) showed that SIM promotes odontogenesis in human dental pulp cells. We hypothesized that SIM could promote DPSC differentiation and mineralization and therefore may have potential use in pulp regeneration.

Several studies that used animal models have found that DPSC implantation in the root canal leads to pulp regeneration (7–12), and research on the regeneration of coronal pulp in pulpotomized teeth would be an interesting pursuit. The aims of our study are the following:

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Regenerative Endodontics

1. To study the effect of SIM on the proliferation and differentiation of DPSCs.

2. To explore the feasibility of DPSC-induced pulp and dentin regeneration after pulpotomy.

Because canine dentition also involves replacement of primary teeth with permanent ones, immature permanent teeth can easily be identified by radiographic monitoring. Therefore, we used the beagle dog model to examine whether DPSC implantation in the pulp chamber can restore tooth integrity in permanent dentition.

Materials and Methods

Animals

Two inbred male beagle dogs aged 20 weeks and weighing 14–16 kg were obtained from Marshall Biotechnology Co Ltd (Beijing, China). This animal study was reviewed and approved by the animal care and use committee of the Medical School of Peking University (No. LA2011-045).

Cell isolation and Culture

With the animals under general anesthesia, the pulp tissues of the canine upper incisors were removed and soaked in Hank’s balanced salt solution. In a biological safety cabinet, the tissues were minced and digested in a solution of 3 mg/mL collagenase type I (Sigma-Aldrich, St Louis, MO) and 4 mg/mL dispase (Sigma-Aldrich) for 1 hour at 37°C. Single-cell suspensions were obtained by passing the cells through a 70-μm strainer (Falcon; Corning Life Sciences, Tewksbury, MA). These suspensions were seeded onto 6-well plates (Costar; Corning) (0.5–1.0 × 10^5well) containing an alpha modification of Eagle medium ( Gibco-Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences, Logan, UT), 2 mmol/L glutamine (Sigma-Aldrich), 100 U/mL penicillin, and 100 mg/mL streptomycin (Sigma-Aldrich), and the plates were incubated at 37°C in 5% CO₂ (13). The growth medium was changed every 2 days until the cells were cloned, and the cells were then transferred into a new dish. When they reached 80% confluence, the cells were again passaged. The cells used in this study were at passage 2.

Odontogenic/Osteogenic Differentiation

The canine DPSCs (cDPSCs) were seeded onto 96-well plastic culture plates at 2.0 × 10^3 cells/well with complete medium. After 24 hours, the medium was changed to complete medium supplemented with SIM (Sigma-Aldrich) (0.01, 0.1, or 1 μmol/L). At 1, 3, and 5 days after SIM addition, the cell counting kit-8 (CCK-8) assay was performed according to the manufacturer’s instructions to evaluate the number of viable cells. The proliferation efficiency was determined by measuring viable cells. The proliferation efficiency was determined by measuring the optical absorbance at a wavelength of 495 nm with a microplate reader (ELx808I; BioTek, Winooski, VT).

For mineralization assays, cDPSCs were seeded onto 6-well plates at 5.0 × 10^3 cells/well, grown to 70% confluence, and incubated for 2 weeks with SIM (0.01, 0.1, or 1 μmol/L)-supplemented differentiation medium containing 10 mmol/L dexamethasone, 10 mmol/L β-glycerophosphate, 50 mg/mL ascorbate phosphate, 10 mmol/L 1,25-dihydroxyvitamin D₃, and 10% fetal bovine serum. The cells were then washed 3 times with phosphate-buffered saline and sonicated with 1% Triton X-100 for 30 minutes on ice. Cellular alkaline phosphatase (ALPase) activity was assayed by using the method reported by Lowry et al (14), with p-nitrophenyl phosphate as the substrate. The enzyme activity is expressed as micromoles of p-nitrophenol produced per milligram of protein. To examine mineral nodule formation, cultured cells were fixed in 4% paraformaldehyde and washed in water, and the mineralization of the extracellular matrix was assayed by 1% alizarin red S staining.

Transplantation

The cDPSCs were cultured with and without SIM (1 μmol/L) for 3 days. Approximately 5.0 × 10^5 cDPSCs (second passage) were mixed with 40 mg hydroxyapatite particles (BioOsteon, Beijing, China) and transplanted subcutaneously into the dorsal surface of four 8- to 10-week-old immunocompromised mice (C3H/SCID; Vitalriver, Beijing, China) according to the method reported by Krebsbach et al (15). Each mouse received 2 different subcutaneous transplants in symmetrical regions. These procedures were performed in accordance with the specifications of an approved animal protocol of the Health Science Center, Peking University (LA2011-045). The transplants were harvested after 8 weeks, fixed with 4% paraformaldehyde, decalcified with buffered 10% EDTA (pH 8.0), rinsed in paraffin, and embedded in paraffin. Sections (5 μm) were deparaffinized and stained with hematoxylin-eosin. DP2-BSW software (Olympus Life Science) was used for quantification of newly formed mineralized tissue in vitro. The rate of observation of mineralized tissue areas was calculated as the area of mineralized tissue in percentage divided by the total area for 4 representative areas from each group.

Pulp Regeneration with SIM-induced cDPSCs as the Pulp-capping Material

Eighteen immature premolars of the 2 inbred 20-week-old male beagle dogs were used in this study. Radiography showed that all the teeth had open apices and no apical periodontal disease. With the dogs under general anesthesia induced by intravenous administration of pentobarbital sodium, the pulp chamber was accessed by using a round carbide bur in a high-speed handpiece. The coronal pulp was removed with a sharp excavator spoon until the root canal orifice was exposed. After bleeding was stopped, the residual pulp was capped with one of the capping materials, as explained later (Table 1).

Once different capping materials were transplanted into the pulp cavity, the cavities were sealed with Fuji IX GIC (Fuji, Japan) and bonded with resin composite (3M-ESPE Dental Supplies, Irvine, CA).

Radiographic examination was conducted every 2 weeks until the root apex closed, which takes 10 weeks. Subsequently, all experimental teeth were extracted, fixed with 4% paraformaldehyde, decalcified with

<table>
<thead>
<tr>
<th>Group</th>
<th>Capping material</th>
<th>Description</th>
<th>Number</th>
</tr>
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<tbody>
<tr>
<td>MTA group</td>
<td>MTA</td>
<td>MTA was capped on the surface of the root pulp (control group).</td>
<td>N=4</td>
</tr>
<tr>
<td>G group</td>
<td>Absorbable gelatin sponge (Gelatamp; Roeko)</td>
<td>Absorbable gelatin sponge was incubated in basic medium for 1 hour at 37°C, and then transplanted into the pulp cavity. Approximately 1.0 × 10^5 of second-passage autologous cDPSCs were co-cultured with absorbable gelatin sponge for 1 hour at 37°C, and then transplanted into the pulp cavity.</td>
<td>N=4</td>
</tr>
<tr>
<td>CG group</td>
<td>cDPSCs + absorbable gelatin sponge</td>
<td>Approximately 1.0 × 10^5 of the expanded second-passage autologous cDPSCs, adding 1 μmol/L SIM, were combined with absorbable gelatin sponge, co-cultured for 1 hour at 37°C, and then transplanted into the pulp cavity.</td>
<td>N=5</td>
</tr>
<tr>
<td>SCG group</td>
<td>SIM + cDPSCs + absorbable gelatin sponge</td>
<td>Approximately 1.0 × 10^5 of the expanded second-passage autologous cDPSCs, adding 1 μmol/L SIM, were combined with absorbable gelatin sponge, co-cultured for 1 hour at 37°C, and then transplanted into the pulp cavity.</td>
<td>N=5</td>
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</tbody>
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buffered 10% EDTA (pH 8.0), and embedded in paraffin. Sections (4 μm) were deparaffinized and stained with hematoxylin-eosin. All samples were sectioned at furcation areas in each experimental group, and data were collected from serial sections. The ratios of the areas showing pulp regeneration to the area of the pulp cavity (hereafter called ratio of regeneration, in percentage) were compared by using DP2-BSW software.

**Statistical Analysis**

Cell proliferation, ALPase activity, and ratio of regeneration were calculated in each group and analyzed by using 1-way analysis of variance. The Student t test was used to compare the area of newly formed mineralized tissues (in percentage) divided by the total area in sections from immunocompromised mice. SPSS software (version 13.0; SPSS, Chicago, IL) was used for all statistical analysis. The mean difference was considered significant at .05 and 95% confidence interval.

**Results**

**Effects of Different Concentrations of SIM on Cell Proliferation and Differentiation**

Cultured cDPSCs were characterized on the basis of their typical fibroblast-like morphology (Fig. 1A). The effects of different concentrations of SIM on cDPSCs proliferation are shown in Figure 1B. At 0.5 μmol/L, SIM had no effects on proliferation until 5 days in culture. However, cDPSCs proliferation was significantly suppressed compared with the control when they were incubated with 1 μmol/L SIM for 3 and 5 days. Furthermore, the number of live cells diminished rapidly in the medium containing greater than 5 μmol/L SIM.

The cDPSCs were treated with different concentrations of SIM for 2 weeks. ALPase activity increased in a dose-dependent manner when cDPSCs were incubated with SIM (*P < .05) (Fig. 1D). The activity was significantly higher in the group treated with 1 μmol/L SIM than in the control group. Mineral nodule formation also

**Figure 1.** *In vitro* proliferation and differentiation of SIM-induced cDPSCs. (A) Cultured cDPSCs were characterized by their typical fibroblast-like morphology. (B) Effects of different concentrations of SIM on cDPSCs proliferation. (C) Alizarin red S staining indicated that SIM-treated cells showed a dose-dependent increase in mineral nodule formation. (D) ALPase activity increased in a dose-dependent manner when cDPSCs were incubated with SIM (*P < .05). OD, optical density.

**Figure 2.** SIM accelerated mineralized tissue formation *ex vivo*. cDPSCs were treated with SIM and then transplanted into immunocompromised mice. Mineralized tissues were formed in both the SIM (B) and control groups (A) (*arrowheads*). The rate of mineralized tissue formation was calculated as the area showing mineralized tissue (in percentage) divided by the total area. Untreated cells only produced 17.4% ± 2.9% mineral tissues, whereas SIM-treated cDPSCs produced 22.8% ± 2.7% (*P < .05*) (C).
increased in a dose-dependent manner, as demonstrated by alizarin red S staining (Fig. 1C).

**SIM Accelerated Mineralized Tissue Formation Ex Vivo**

The cDPSCs were treated with SIM and then transplanted into immunocompromised mice. Mineralized tissues were formed in both the test and control groups after 8 weeks. To evaluate the effects of SIM on mineralized tissue formation, the rate of formation of mineralized tissue areas was calculated as the mineralized tissue area in percentage divided by the total area for 4 representative areas from each group. The cDPSCs pretreated with SIM produced 22.8% ± 2.7% mineral tissues, but untreated cells only produced 17.4% ± 2.9% (P < .05) (Fig. 2). These *ex vivo* tests demonstrated that SIM promotes cDPSCs mineralization.

**SIM-pretreated cDPSCs Induced Coronal Pulp Regeneration in the Pulpotomy Model**

Eighteen immature premolars with open apices from 2 inbred beagle dogs were used in this study. The coronal pulp was removed, and different capping materials were transplanted on the residual root pulp. After a 10-week observation period, the teeth continued to develop, as indicated by closure of the root apex and thickening of the root canal wall observed on radiographic examination (Fig. 3).

Histologically, the 4 treatment groups showed different degrees of regeneration (Fig. 4). In the mineral trioxide aggregate (MTA) group, a dental bridge was formed right at the root canal orifice, and the pulp below looked like natural pulp. Examination under magnification showed that odontoblastic cells were connecting the pulp and dental bridge. In the G group, which received an absorbable gelatin sponge cap, a small amount of pulp tissue was generated toward the pulp cavity, and calcification was seen in this area. The ratio of pulp regeneration was 47.3% ± 2.5%. In the CG group, in which cDPSCs were introduced into the pulp cavity, more pulp tissues were generated around that newly regenerated dentin in conjunction with the primary dentin, and the ratio of regeneration was 76.8% ± 4.3%. In the SCG group, in which cDPSCs pretreated with SIM were introduced into the pulp cavity, regenerated pulp was found filling nearly the entire pulp cavity. Newly formed dentin deposits succeed to the primary dentin continuously, and odontoblastic cells could be seen in the regenerated area. The ratio of regeneration was 85.8% ± 0.9%, which was significantly higher than that in all other groups (P < .05). Histologic findings showed apical closure in all groups, and the root pulp in all groups was the same as natural pulp.

**Discussion**

According to previous research, DPSCs are capable of regenerating dental structures (8,16–19). SIM is known to enhance bone formation by accelerating BMP-2 expression, and studies have shown that it also acts on DPSCs (4, 5). In the present study, a cell proliferation assay that was based on the CCK-8 marker showed that 1 μmol/L SIM suppressed DPSC growth slightly. However, SIM at a lower concentration had no effects on DPSC proliferation, and at a higher concentration, it led to cell death. Similar phenomena were
also observed with other cell types, including osteoblastic cells, vascular smooth muscle cells, and neuronal cells (20–22).

In a previous study, statin was found to be a strong accelerator of dspp gene expression (4). To examine the effect of SIM on mineralization, cells treated with 3 concentrations of SIM were tested for ALPase activity and alizarin red S staining. ALPase activity, an early marker of mineralization, is enhanced when DPSCs are tending toward odontoblastic differentiation. Cells cultured with 1 μmol/L SIM for 2 weeks had significantly higher ALPase activity than those cultured with 0.1 μmol/L SIM or the control. Alizarin red S staining helps measure mineralization ability in vitro. Furthermore, cells cultured in odontogenic medium with 1 μmol/L SIM for 2 weeks produced the most mineral nodules. Our results indicated that the ideal concentration of SIM for DPSCs proliferation and mineralization was 1 μmol/L, and this concentration was used in the subsequent experiments.

Regenerative medicine, of which pulp regeneration forms an important part, is based on stem cells, signals, and scaffolds (23, 24). Dental pulp tissue has the potential to regenerate dentin in response to hazardous stimuli. Previous studies showed that implantation of stem cell scaffolds into the empty root canal could induce regeneration of pulp-like tissue (11, 18). However, few studies have focused on teeth with partially lost coronal pulp (25–28).

Clinically, pulpotomy can be used to treat early pulpitis, and MTA is widely used for direct pulp capping and pulpotomy because it is known to induce hard tissue repair (29–31). In the present study, all 4 groups showed increased root length and thickness, and apex closure was

**Figure 4.** Coronal pulp regeneration after pulpotomy in beagle dog premolars at 10 weeks after transplantation of different capping materials (Table 1) (hematoxylin-eosin staining). The pulp cavity is marked by dotted lines. (A and E) MTA group. MTA was the capping material; a dental bridge was formed right at the root canal orifice (arrows). High magnification (E) showed that odontoblastic cells connected the pulp and dental bridge. (B and F) G group. An absorbable gelatin sponge cap was the capping material; a small amount of pulp tissue was generated toward the pulp cavity (regenerated ratio: 47.3% ± 2.5%), and calcification was seen in this area. (C and G) CG group. cDPSCs were used as the capping material; more pulp tissues were generated (regenerated ratio: 76.8% ± 4.3%), and newly regenerated dentin could be seen in conjunction with the primary dentin. (D and H) SCG group. cDPSCs pretreated with SIM were introduced into the pulp cavity, and regenerated pulp was found to have filled nearly the entire pulp cavity (regenerated ratio: 85.8% ± 0.9%). Newly formed dentin deposits covered the primary dentin continuously (arrows), and odontoblastic cells could be seen in the regenerated area. All groups showed apical closure, and the root pulp was the same as natural pulp. (I) Histogram of ratios of regeneration in the G, CG, and SCG groups. Data are given as means ± standard deviation; statistical analysis was performed by using 1-way analysis of variance, *P < .05.
observed on radiography. Furthermore, all groups showed continued root development. In the MTA group, a dentin bridge was formed at the root canal orifice, which kept the regenerated pulp from entering the pulp chamber. Therefore, solely an MTA cap is not suitable, and another alternative must be sought. An absorbable gelatin sponge is a commonly used collagen scaffold. In teeth extraction surgery, an absorbable gelatin sponge is usually placed into the socket of the extracted tooth to reduce the possibility of complications (hemorrhage, infection, pain, etc). Our previous studies showed that cDPSCs were easily absorbed onto the absorbable gelatin sponge (19). In the G and CG groups, in which the absorbable gelatin sponge with or without cDPSCs, respectively, was introduced into the pulp chamber, regenerated pulp tissues were seen where the original pulp had been removed. Furthermore, the group in which absorbable gelatin sponge mixed with cDPSCs was introduced into the pulp chamber (CG group) showed a regeneration area of 76.8% ± 4.3%, which was significantly higher than that observed in the group that received absorbable gelatin sponge alone (G group, regeneration area: 47.3% ± 2.5%). The observed tissue regeneration indicated that exogenous cDPSCs can differentiate into pulp cells and subsequently to pulp tissues or stimulate residual pulp to proliferate and migrate to the pulp chamber.

In the SCG group, SIM-treated cDPSCs were introduced into the experimental teeth. In the hematoxylin-eosin staining result, pulp tissue and migrate to the pulp chamber. Subsequently to pulp tissues or stimulate residual pulp to proliferate and arrangement of root pulp cells were like natural pulp in all 4 groups. Collectively, the results indicated that introduction of SIM-treated cDPSCs into the pulp chamber after pulpotomy in beagle dogs could lead to coronal pulp regeneration as well as dentin regeneration effectively and rapidly. We also concluded that SIM stimulates DPSC mineralization both in vitro and in vivo. SIM-treated cDPSCs showed increased ALPase activity and mineral nodule formation. Furthermore, transplantation experiments with immunocompromised mice demonstrated that SIM increased the odontogenic differentiation of cells ex vivo. Thus, stem cell therapy seems to show promise in dentin regeneration, and SIM is a favorable biocompatible pulp-capping material that can induce pulp regeneration and de novo dentin formation.

Acknowledgments
Weiqian Jia and Yuming Zhao contributed equally to this study as first authors.

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

References
5. Min KS, Lee YM, Hong SO, et al. Simvastatin promotes odontoblastic differentiation and expression of angiogenic factors via heme oxygenase-1 in primary cultured hu-
11. Ishizaka R, Iohara K, Murakami M, et al. Regeneration of dental pulp following pul-
pectomy by fractionated stem/progenitor cells from bone marrow and adipose tis-
tors of trabecular bone and osteogenic differentiation of bone marrow mesen-
30. Asgary S, Shirani A, Fattah MB. MTA and ferric sulfate in pulpotomy outcomes of pri-