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

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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, canine DPSCs (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% $\pm$ 2.5%, 76.8% $\pm$ 4.3%, and 85.8% $\pm$ 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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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 Hanks' 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-mm strainer (Falcon; Corning Life Sciences, Tewksbury, MA). These suspensions were seeded onto 6-well plates (Costar; Corning) ($0.5–1.0 \times 10^3$/well) 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% CO_2 (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% confluency, 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 the optical absorbance at a wavelength of 495 nm with a microplate reader (ELx808IU; BioTek, Winooski, VT).

For mineralization assays, cDPSCs were seeded onto 6-well plates at 5.0×10^4 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 nmol/L dexamethasone, 10 mmol/L β-glycerophosphate, 50 mg/mL ascorbate phosphate, 10 nmol/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 of Lowry et al (14), with p-nitrophenyl phosphate as the substrate. The enzyme activity is expressed as micromoles of p-nitrophenyl 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 5.0×10^6 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 (CB-17 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), and then 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 vivo*. 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 1. Capping Materials in Different Groups

Group	Capping material	Description	Number
MTA group	MTA	MTA was capped on the surface of the root pulp (control group).	N = 4
G group	Absorbable gelatin sponge (Gelatamp; Roeko)	Absorbable gelatin sponge was incubated in basic medium for 1 hour at 37°C and then transplanted into the pulp cavity.	N = 4
CG group	cDPSCs + absorbable gelatin sponge	Approximately 1.0×10^7 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.	N = 5
SCG group	SIM + cDPSCs + absorbable gelatin sponge	Approximately 1.0×10^7 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.	N = 5