Biological Analysis of Simvastatin-releasing Chitosan Scaffold as a Cell-free System for Pulp-dentin Regeneration

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Abstract

Introduction: The improvement of biomaterials capable of driving the regeneration of the pulp-dentin complex mediated by resident cells is the goal of regenerative dentistry. In the present investigation, a chitosan scaffold (CHSC) that released bioactive concentrations of simvastatin (SIM) was tested, aimed at the development of a cell-free tissue engineering system. **Methods:** First, we performed a dose-response assay to select the bioactive dose of SIM capable of inducing an odontoblastic phenotype in dental pulp cells (DPCs); after which we evaluated the synergistic effect of this dosage with the CHSC/DPC construct. SIM at 1.0 μmol/L (CHSC-SIM1.0) and 0.5 μmol/L were incorporated into the CHSC, and cell viability, adhesion, and calcium deposition were evaluated. Finally, we assessed the biomaterials in an artificial pulp chamber/3-dimensional culture model to simulate the cell-free approach *in vitro*. **Results:** SIM at 0.1 μmol/L was selected as the bioactive dose. This drug was capable of strongly inducing an odontoblastic phenotype on the DPC/CHSC construct. The incorporation of SIM into CHSC had no deleterious effect on cell viability and adhesion to the scaffold structure. CHSC-SIM1.0 led to significantly higher calcium-rich matrix deposition on scaffold/dentin disc assay compared with the control (CHSC). This biomaterial induced the migration of DPCs from a 3-dimensional culture to its surface as well as stimulated significantly higher expressions of alkaline phosphatase, collagen type 1 alpha 1, dentin matrix acidic phosphoprotein 1, and dentin sialophosphoprotein on 3-dimensional–cultured DPCs than on those in contact with CHSC. **Conclusions:** CHSC-SIM1.0 scaffold was capable of increasing the chemotaxis and regenerative potential of DPCs. *(J Endod 2018;■:1–6)*

Key Words

Cell differentiation, dental pulp, scaffolds, tissue engineering

Significance

This study showed the chemotactic and bioactive potentials of chitosan scaffolds associated with low-dose simvastatin on dental pulp cells. Therefore, this technology may be considered a promising strategy as a cell-free system for pulp-dentin complex regeneration.

With the advances in tissue engineering and regenerative dentistry, efforts have been directed to the development of bioactive scaffolds for pulp-dentin complex regeneration. The design of biomaterials in combination with the controlled release of signaling clues to direct the fate of mesenchymal stem cells (MSCs) into osteo/odontoblastic phenotypes is a highly promising therapeutic strategy to achieve dental and craniofacial mineralized tissue engineering (1–3). To guide adequate tissue regeneration, desirable aspects of scaffold chemistry should be addressed, including the creation of a polymeric network that mimics extracellular matrix components, allowing cell interaction to occur (4).

Porous chitosan scaffolds (CHSCs), which possess polymeric chains structurally similar to glycosaminoglycan (GAG), have been reported to be a suitable substrate for dental pulp cell (DPC) adhesion, proliferation, and odontoblastic differentiation upon adequate cell signaling (5). Chitosan has generated great interest in the tissue engineering field because of its good biocompatibility, biodegradability into nontoxic components, protein affinity, and hemostatic and antimicrobial potential (6, 7). Therefore, CHSCs might be useful in direct pulp capping therapy because they may provide a biocompatible matrix for the adhesion, proliferation, and differentiation of endogenous DPCs into odontoblastlike cells capable of depositing and mineralizing dentin matrix, thus restoring dentin integrity at the pulp-dentin border.

The release of bioactive substances for a direct influence on the behavior of ingrowing cells on the scaffold structure is considered a hallmark of tissue engineering applications, thus increasing the amount and quality of neotissue genesis in a shorter time (4). Researchers have found that local application of statins is capable of strongly accelerating the repair of bone defects *in vivo* (8, 9). Recent review articles regarding this specific topic concluded that local delivery of simvastatin (SIM) from biomaterials seems to be more reliable than systemic administration for bone regeneration; however,

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depending on the released dosage, SIM can either accelerate or retard mineralized neotissue genesis (10, 11). At low concentrations, these drugs feature pleiotropic effects with mesenchymal stem cells, increasing the expression of several osteo/odontoblastic markers, such as dentin sialophosphoprotein (DSPP), dentin matrix acidic phosphoprotein 1 (DMP-1), alkaline phosphatase (ALP), collagen type 1 alpha 1 (Col1A1), osteocalcin, osteopontin, runt-related transcription factor 2, and bone morphogenetic protein 2 (BMP-2), leading to intense mineralized matrix deposition *in vitro* (12–15). Accordingly, the objective of this study was to develop a porous chitosan scaffold capable of releasing SIM at a bioactive level, stimulating the migration and odontoblastic phenotype expression of DPCs, aimed at the development of a cell-free tissue engineering system for regeneration of the pulp-dentin complex. The tested hypothesis was that a chitosan scaffold associated with a low dose of simvastatin can induce DPC migration and odontoblastic differentiation.

Materials and Methods

Establishment of DPCs

Primary culture of DPCs was obtained by enzymatic dissociation of fresh pulp tissue from sound human third molars from a 24-year-old male patient as described in detail by Soares et al (5). The patient provided informed consent according to the Declaration of Helsinki (protocol #30939314.5.0000.5416), and the study was approved by the Research Ethics Committee of the Araraquara School of Dentistry, Universidade Estadual Paulista, São Paulo, Brazil. The cells were cultured in complete alpha minimum essential medium (α-MEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, L-glutamine, and 1% penicillin-streptomycin and used from passages 3 to 6 for the experimental protocols.

Selecting Bioactive Dosages of Simvastatin

The DPCs were seeded on 96-well plates (Corning, Corning, NY) (1×10^4 cells/well) in complete α-MEM and incubated for 24 hours. They were then cultured in 100 μL osteogenic medium (complete α-MEM plus 50 μg/mL ascorbic acid and 5 mmol/L β-glycerophosphate; Sigma-Aldrich, St Louis, MO) supplemented with 0 μmol/L, 0.01 μmol/L, 0.1 μmol/L, or 1 μmol/L SIM (Sigma-Aldrich) for up to 21 days. The culture medium supplemented or not with SIM was replaced every 48 hours. The metabolic activity of cells (alamarBlue Assay; Thermo Fisher Scientific, Waltham, MA), ALP activity (thymolphthalein monophosphate substrate–based assay), calcium deposition (alizarin red), and cell migration (wound healing and transwell assays) were evaluated (5, 16). Detailed information can be found in the Supplementary Material.

Synergistic Potential of SIM and CHSCs

A 2% chitosan solution (75%–85% deacetylated, mol wt 310,000–375,000 d; Sigma-Aldrich) in 2% aqueous solution of glacial acetic acid (Sigma-Aldrich) was poured (500 μL) into polystyrene tubes, frozen at -80°C for 4 hours, and freeze-dried at -56°C overnight to obtain CHSCs. The surface and transversal slices of CHSCs were evaluated using scanning electron microscopy (12–15 kV [JMS-6610V Scanning Microscope; JEOL, Tokyo, Japan]). The overall porosity was calculated by ImageJ software (National Institutes of Health, Bethesda, MD) in 3 samples at 100× magnification ($n = 3$), and the CHSC featured a mean porosity of 35.1% ± 1.9%.

CHSC samples (5-mm diameter and 1-mm thickness) were sterilized in 70% ethanol (30 minutes) under a vacuum, washed in phosphate-buffered saline (PBS) (3 times of 30 minutes), and incubated in complete α-MEM overnight at 37°C and 5% CO_2. The cells (1×10^5 cells) in 3 μL α-MEM were seeded onto the scaffolds followed by incubation for 30 minutes to allow cells to adhere exclusively to the scaffold structure and cultivation in 500 μL α-MEM for 24 hours. Thereafter, the DPC/scaffold constructs were cultivated on osteogenic medium supplemented or not with 0.1 μmol/L SIM for up to 21 days. The following groups were established: CHSC/DPC construct cultivated with osteogenic medium and CHSC + SIM/DPC construct cultivated with osteogenic medium supplemented with 0.1 μmol/L SIM. Cell viability (Live/Dead Assay, Invitrogen), proliferation (alamarBlue Assay), cell spread (F-actin staining), ALP activity (p-nitrophenyl phosphate substrate), mineralized matrix deposition (alizarin red), and cell migration (transwell) were evaluated at different time points (5, 16).

Bioactive Potential of SIM–releasing CHSCs

The CHSC was immersed in PBS (pH = 7.4) with 15% dimethyl sulfoxide (Sigma-Aldrich) with or without 1 mmol/L SIM for 24 hours at 37°C in order to calculate the percentage of drug release. After the determination of 10% SIM release within 24 hours, 1.0 μmol/L (SIM1.0) and 0.5 μmol/L (SIM0.5) SIM were incorporated into CHSCs, aimed at the release of 0.1–0.05 μmol/L SIM. The cell viability (Live/Dead assay), spread (F-actin staining), and calcium-rich mineral deposition (alizarin red) were evaluated on DPCs seeded on CHSC, CHSC-SIM1.0, and CHSC-SIM0.5 adapted to circular perforations (1-mm thick and 4-mm diameter) prepared on human dentin discs (2-mm thick and 8-mm diameter, protocol #30939314.5.0000.5416) (17).

To evaluate the potential of the biomaterials as a cell-free approach for dentin regeneration, we prepared an *in vitro* model of an artificial pulp chamber (APC) with 3-dimensional (3D)-cultured DPCs. Human dentin discs (1-mm thick and 8-mm diameter) containing a central perforation (1-mm thick and 4-mm diameter) were adapted to the APC between 2 silicon O-rings (5). The 3D matrix, composed of 1:1 Hydromatrix (Sigma-Aldrich) and type 1 collagen (rat tail, 3.67 mg/mL; Corning), was pipetted onto the pulpal sides of dentin discs (100 μL). After pH neutralization, 1×10^5 DPCs were seeded on the 3D matrix, and 24 hours thereafter the scaffold (5-mm diameter and 1-mm thick) was adapted to the dentin discs in intimate contact with the 3D cell culture. The set was incubated in complete α-MEM (with no osteogenic supplementation) for 21 days, and the presence of viable cells on the 3D matrix and scaffold surfaces was evaluated by the Live/Dead assay (5). The gene expression of ALP, Col1A1, DSPP, and DMP-1 was evaluated in the 3D culture by real-time polymerase chain reaction at the 21-day period (5). Detailed information can be found in the Supplementary Material.

Statistical Analysis

The experiments were performed twice to ensure reproducibility. Data were compiled and normalized by a negative control group (no SIM supplementation). Data were then analyzed using Student t tests and 1- or 2-way analysis of variance complemented by the Tukey test for observation of the significant differences between the study groups. A value of $P < .05$ was considered to be statistically significant.

Results

Bioactive Potential of Low-dose SIM with DPCs

Regarding cell viability, no significant difference was observed among groups on 1 day; however, a significant reduction in the proliferative capability was observed for the cells cultivated in contact with SIM at 7 and 14 days in comparison with the negative control (Fig. 1*A*). The dose response for SIM inducing odontoblastic phenotypes on DPCs showed that 0.1 μmol/L SIM featured a bioactive