Concentration-dependent Effect of Sodium Hypochlorite on Stem Cells of Apical Papilla Survival and Differentiation

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Abstract

Introduction: Intracanal disinfection is a crucial step in regenerative endodontic procedures. Most published cases suggest the use of sodium hypochlorite (NaOCl) as the primary irrigant. However, the effect of clinically used concentrations of NaOCl on the survival and differentiation of stem cells is largely unknown. In this study, we tested the effect of various concentrations of NaOCl on the stem cells of the apical papilla (SCAPs) survival and dentin sialophosphoprotein (DSPP) expression. **Methods:** Standardized root canals were created in extracted human teeth and irrigated with NaOCl (0.5%, 1.5%, 3%, or 6%) followed by 17% EDTA or sterile saline. SCAPs in a hyaluronic acid–based scaffold were seeded into the canals and cultured for 7 days. Next, viable cells were quantified using a luminescence assay, and DSPP expression was evaluated using quantitative real-time polymerase chain reaction. **Results:** There was a significant reduction in survival and DSPP expression in the group treated with 6% NaOCl compared with the untreated control group. Comparable survival was observed in the groups treated with the lower concentrations of NaOCl, but greater DSPP expression was observed in the 1.5% NaOCl group. In addition, 17% EDTA resulted in increased survival and DSPP expression partially reversing the deleterious effects of NaOCl. **Conclusions:** Collectively, the results suggest that dentin conditioning with high concentrations of NaOCl has a profound negative effect on the survival and differentiation of SCAPs. However, this effect can be prevented with the use of 1.5% NaOCl followed by 17% EDTA. The inclusion of this irrigation regimen might be beneficial in regenerative endodontic procedures. *(J Endod 2014;40:51–55)*

Key Words

Dentin conditioning, dentin sialophosphoprotein, EDTA, gene expression, irrigant, regenerative endodontics, stem cells of apical papilla, sodium hypochlorite, survival, viability

The field of endodontics has seen a shift toward biologically based regeneration of pulpal tissues. Regenerative endodontic procedures rely on adequate disinfection while creating a microenvironment conducive for stem cell survival and differentiation (1). In current regenerative endodontic procedures, mesenchymal stem cells (MSCs) are delivered from the periradicular tissues into the root canal space via evoked bleeding (2). Postnatal MSCs include the dental pulp stem cells (DPSCs), stem cells from human exfoliating deciduous teeth (SHEDs), periodontal ligament stem cells, dental follicle progenitor cells, inflammatory periapical progenitor cells, and stem cells from the apical papilla (SCAPs) (3–6). Of special interest to endodontics are SCAPs. These cells can be found in the apical papilla immediately adjacent to the apex of the root canal, allowing for an easier incorporation of SCAPs in regenerative endodontic procedures (1, 2). SCAPs have also been reported to have high proliferation rates and odontogenic differentiation capacity (4).

Multiple case reports of regenerative endodontic procedures have been published, with significant differences in disinfection protocols. These have varied from the use of 5.25% sodium hypochlorite (NaOCl) followed by saline and 0.12% chlorhexidine (1, 7, 8) 5.25% NaOCl, or 2.5% NaOCl alone to 6% NaOCl followed by 2% chlorhexidine (1, 9). Although proven to show effectiveness as root canal irrigants for bactericidal, bacteriostatic, and tissue dissolution properties (10, 11), these irrigants have also been shown to be cytotoxic to fibroblasts, periodontal ligament stem cells, HeLA cells, SHEDs, and SCAPs (12–16). When NaOCl is used at 6%, it has a profound detrimental effect on SCAP survival using an organotype human root model (12) and on odontoblastic differentiation of DPSCs *in vivo* (17). Thus, dentin treatment with 6% NaOCl has a negative effect on the survival and differentiation of stem cells when in contact with the conditioned dentin.

Nonetheless, NaOCl is the most commonly used endodontic irrigant, and it has been used in the great majority of all the regenerative/revascularization reported cases (8, 18, 19). However, the effect of various clinically relevant concentrations of NaOCl on the survival and odontoblastic differentiation of stem cells has never been previously investigated. Thus, the aim of this study was to evaluate the effect of different concentrations of NaOCl on the survival and odontoblastic differentiation of SCAPs in an organotype root canal model.

Methods

Patient Recruitment

This study was approved by the Institutional Review Board of the University of Texas Health Science Center at San Antonio, San Antonio, Texas. Third molar teeth diagnosed with vital pulp but with an indication for extraction were collected from the clinics

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of the University of Texas Health Science Center at San Antonio School of Dentistry upon informed consent.

Extracted teeth were immediately placed in ice-cold Hank's Balanced Salt Solution (HBSS; Sigma-Aldrich, St Louis, MO). Gingival and periodontal tissue was removed from the tooth surface with a sterile surgical blade. Teeth were then cleaned with iodine and 70% ethanol to prevent contamination followed by 5 washes with cold phosphate-buffered saline. Sterilized high-speed burs were used to section roots to achieve standardized segment lengths of 5 mm. The root canals were instrumented under constant sterile saline irrigation to a constant diameter of 1.3 mm using #130 LSX files (Sybron Endo, Orange, CA), simulating an open/immature apex often observed in regenerative cases. Thus, nontapered LSX instrumentation was used to prepare a parallel-walled canal space with a constant 1.3-mm diameter while maintaining root structural integrity. All root segments were air-dried, gas sterilized with ethylene oxide, and maintained at room temperature until use.

A previously characterized SCAP line was used in all experiments (20). Cells were cultured and expanded by adding single-cell suspensions (1×10^5 cells) in media composed of alpha-minimum essential medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine, 1 L-glutamine (Gemini, West Sacramento, CA), penicillin (100 U/mL, Gemini), and streptomycin (100 mg/mL, Gemini) to 10-cm cell culture dishes. Cells were allowed to expand in culture to 70%–80% confluency followed by treatment with 0.05% trypsin (Gibco, Carlsbad, CA) and pass the culture to subsequent culture plates or used in experiments. The cell suspension concentration was determined by a TC10 automated cell counter (Bio-Rad, Hercules, CA). SCAPs between the third and eighth passages were used in this study.

Scaffold (Hyaluronic Acid Hydrogel)

A polymer pregel solution containing type I rat tail collagen (BD Biosciences, San Jose, California), laminin (1.5 mg/mL; Trevigen, Gaithersburg, MD), and hyaluronic acid (HA) (1.5 mg/mL; molecular weight = 1,500 kDa; Sigma-Aldrich) were made in Dulbecco's phosphate-buffered saline (Sigma-Aldrich). We diluted the collagen stock solution in 0.2% acetic acid to obtain the final concentration of 1.5 mg/mL. Collagen, 10× HBSS, and 1× HEPES solutions were mixed together in an 8:1:1 composition solution. The pregel was kept at 4°C until use.

Organotype Root Canal Model

Root tips were placed in HBSS to allow for dentin rehydration for at least 10 minutes. Each root tip was secured in a mechanical vise, and an 18-G needle was positioned at the coronal-most opening of the canal. The needle was connected to syringes containing different concentrations of NaOCl (0.5%, 1.5%, 3%, or 6%) or control (17% EDTA). The syringes were placed in a KD230 computer-controlled syringe pump (KD Scientific, Hayward, CA) programmed to deliver 2 mL/min flow of irrigants through the canal. Each sample was irrigated with NaOCl for a total of 10 minutes (a 20-mL total volume). Next, samples were irrigated with 10 mL EDTA or saline for 5 minutes followed with a final flush of sterile saline (20 mL) to remove residual irrigant. As a control, sodium thiosulfate (5%, 10 mL) was used to neutralize any potential residual effect of the highest concentration of NaOCl (6%) followed by removal with saline. All segments were blotted dry with sterile paper towels before seeding of the stem cells in HA-based scaffolds.

A total of 2.5×10^5 SCAPs in 2 μL was mixed with 13 μL HA-based hydrogel solution, immediately pipetted into the root canals, and placed into 24-well plates. Alternatively, cells at different concentrations were added to the scaffold to serve as the control and to generate a quantification standard curve. All samples were then incubated at 37°C and 5% CO_2 for 30 minutes to allow for the polymerization of the hydrogel followed by the addition of media (described previously) and incubation for 7 days. At the end of the incubation period, the total cell number was estimated by a CellTiter-Glo Luminescent Cell Vitality Assay (Promega, Madison, WI) or processed for RNA isolation and quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR).

Viable SCAP Quantification

SCAPs at a dynamic concentration range (5×10^4, 1×10^5, 2×10^5, 4×10^5, 8×10^5, and 1×10^6 cells/15 μL) were encapsulated into the HA-based scaffold, incubated for 30 minutes to allow for polymerization of the hydrogel, and quantified using the CellTiter-Glo assay to determine the efficiency of encapsulation and to establish a standard curve for the determination of the total viable cells in the experimental samples.

Briefly, known concentrations of SCAPs in suspension or encapsulated in the HA-based hydrogel were mixed directly with the CellTiter-Glo luminescence reagent as described by the manufacturer. At the same time, the samples were flushed out of the root canals by repeated pipetting of the CellTiter-Glo reagent though the lumen. All the samples were allowed to incubate at room temperature for 30 minutes followed by luminescence measurement in a FlexStation 3 Multi-mode Microplate Reader (Molecular Devices, Sunnyvale, CA).

RNA Isolation and Quantitative Real-time Reverse-transcription Polymerase Chain Reaction

The total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA), and any potential genomic DNA contamination was removed by DNAse treatment (DNA-free kit; Ambion, Austin, TX) under a standardized protocol.

The total RNA samples were used as a template in 1-step real-time polymerase chain reactions using the TaqMan One-Step RT-PCR Master Mix Kit (Life Technologies, Carlsbad, CA) and TaqMan gene expression assays for dentin sialophosphoprotein (DSPP) (assay #Hs00171962) and the endogenous reference glyceraldehyde 3-phosphate dehydrogenase (assay #Hs02758991_g1) (Life Technologies). The relative DSPP gene expression was determined using the comparative delta-delta cycle threshold method ($\Delta\Delta$Ct) using the control group as the calibrator as previously reported (21).

Data Analysis

All experiments for both cell viability quantification and gene expression were repeated at least 3 times (total of $n = 9$/group). Data were analyzed with 1-way analysis of variance with the Bonferroni post hoc test, and significance was set at $P < .05$ by using the Graph Pad Prism 5.0 version software (Graph Pad, La Jolla, CA).

Results

Irrigation with saline only yielded a total count of 3.8×10^4 cells/samples (no treatment control) after 7 days of culturing. Dentin conditioning with 17% EDTA resulted in a 35% increase in SCAPs (survival-positive control). However, dentin conditioning with NaOCl evoked a concentration-dependent decrease in SCAP viability. The concentrations of 0.5%, 1.5%, and 3% all evoked a similar reduction of approximately 37% in SCAP survival, whereas treatment with 6% NaOCl resulted in greatly diminished SCAP survival (5,600 ± 5,500 cells). The addition of a final irrigation with 17% EDTA reversed the negative effects of NaOCl on SCAP survival, resulting in survival comparable with controls (no treatment) but still lower than the survival positive control ($P < .05$)