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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 NaOCI on the survival and differentiation of stem cells is largely unknown. In this study, we tested the effect of various concentrations of NaOCI 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 NaOCI (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% NaOCI compared with the untreated control group. Comparable survival was observed in the groups treated with the lower concentrations of NaOCI, but greater DSPP expression was observed in the 1.5% NaOCI group. In addition, 17% EDTA resulted in increased survival and DSPP expression partially reversing the deleterious effects of NaOCI. Conclusions: Collectively, the results suggest that dentin conditioning with high concentrations of NaOCI has a profound negative effect on the survival and differentiation of SCAPs. However, this effect can be prevented with the use of 1.5% NaOCI 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 phosphatebuffered 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 $(1 \times 10^5$ 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, $10 \times$ HBSS, and $1 \times$ 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 NaOCI (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 NaOCI 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 NaOCI (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 μ L was mixed with 13 μ L 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% CO₂ 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 Cell-Titer-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 × 10⁴, 1 × 10⁵, 2 × 10⁵, 4 × 10⁵, 8 × 10⁵, and 1 × 10⁶ cells/15 μ L) 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 (survivalpositive 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)



Figure 1. NaOCl decreases the SCAP survival. SCAP (25,000 cells) were encapsulated into a HA-based hydrogel and seeded into an organotype root canal model previously irrigated with various concentrations of NaOCl followed by a final irrigation of 17% EDTA or saline. Samples were cultured for 7 days, and the number of viable cells were quantified with a luminescence assay (n = 9-12, experiments were performed in triplicates). Data are presented as the mean \pm standard error of the mean. *P < .05 and **P < .01 as tested by 1-way analysis of variance (Bonferroni post hoc test) compared with the control (untreated canals, *black bar*).

(Fig. 1). Importantly, the neutralization of NaOCI (6%) with sodium thiosulfate (5%) did not reverse the detrimental effects on SCAP survival (data not shown).

Dentin conditioning with 17% EDTA promoted the expression of the odontoblast-like cell marker DSPP, increasing its gene expression by 2.2-fold. On the other hand, NaOCl at the concentration of 1.5% had no effect on DSPP gene expression compared with the control group, whereas the 3% concentration reduced DSPP gene expression by approximately 50%. Importantly, dentin conditioning with 6% NaOCl completely abolished DSPP expression (Fig. 2). Furthermore, the addition of a final irrigation with 17% EDTA increased DSPP expression after 1.5% NaOCl compared with control. A final irrigation with 17% EDTA reversed the deleterious effects of dentin conditioning with 3% and 6% NaOCl, complete and partial reversal, respectively (Fig. 2).

Discussion

NaOCl remains the most widely used disinfection solution in endodontics. It has excellent bactericidal efficacy (22-24) and tissue dissolution capacity (25-27) and serves as a lubricant for endodontic instruments. The first 2 beneficial properties are crucial for the disinfection of immature teeth in regenerative endodontic procedures (REPs), which typically involve minimal to no mechanical preparation (28).

The findings presented in this study confirmed the initial report that dentin treatment (conditioning) with 6% NaOCl was not conducive for SCAP survival in an organotype root canal model (12). Similarly, this study also confirmed that 17% EDTA promotes the survival of SCAPs (12). Importantly, the findings in this study are not related to the residual direct chemical effect on SCAPs because all root canals received profuse irrigation with physiological saline at the end of the irrigation procedure. In addition, control experiments were conducted in which 5% sodium thiosulfate was used in an attempt to block the profound effect of 6% NaOCl dentin treatment on SCAP survival. This



Figure 2. NaOCl decreases the expression of the odontoblastic marker DSPP. SCAPs (25,000 cells) were encapsulated into a HA-based hydrogel and seeded into an organotype root canal model previously irrigated with various concentrations of NaOCl followed by a final irrigation of 17% EDTA or saline. Samples were cultured for 7 days, and DSPP expression compared with the control (untreated canals, *black bar*) was determined by qRT-PCR after normalization with the endogenous reference glyceraldehyde 3-phosphate dehydrogenase (n = 9-12, experiments were performed in triplicates). Data are presented as the mean \pm standard error of the mean. *P < .05, **P < .01, and ***P < .001 as tested by 1-way analysis of variance (Bonferroni post hoc test) compared with the control (untreated canals, *black bar*).

neutralization step failed to prevent the significant reduction of SCAP survival by 6% NaOCl (data not shown). Thus, the effects observed in this study are related to an indirect effect of chemical irrigation on dentin, resulting in a microenvironment that was hostile for SCAP survival as opposed to a direct chemical cytotoxicity.

Clinicians often face the challenge of adequately debriding large infected root canals in REPs. In these procedures, similarly to conventional endodontic therapy, microbial control is crucial; yet, these canals have compromised fragile underdeveloped dentinal walls representing a contraindication for mechanical instrumentation. Chemical debridement remains the main form of disinfection in REPs (1). Thus, lower concentrations of NaOCl were further tested in this study to serve as guidance in selecting the NaOCl concentrations that retain its superb debridement properties while being conducive to the survival of undifferentiated MSCs (such as SCAPs) that are known to be brought into root canals after the disinfection step in REPs (2). We found that dentin treatment with lower concentrations of 0.5%, 1.5%, and 3% of NaOCl resulted in similar reductions in SCAP survival but with significantly better survival than with 6% NaOCl. The 0.5% NaOCl group was not further evaluated in the odontoblastic differentiation experiments because it did not promote greater SCAP survival, and greater concentrations of NaOCl are known to enhance its tissue dissolution and antimicrobial effects (25, 29, 30). Importantly, 17% EDTA completely reversed the effects of the lower concentrations of NaOCl and only partially reversed the negative effect of 6% NaOCl on the survival of SCAPs.

The survival and viability of MSCs in debrided canals are paramount to the goal of REPs. However, in addition to survival, these cells must retain their differentiation capacity. One of the main goals of REPs is continued root development. Thus, the maximization of odontogenic potential in surviving MSCs remains one of the challenges to be overcome. It has been shown that dentin-conditioning with 6% NaOCl

Basic Research—Biology

prevented the differentiation of SHEDs and DPSCs into an odontoblastlike phenotype in vitro (31) and in vivo (17, 31). Importantly, 17% EDTA promoted the differentiation of DPSCs into odontoblast-like cells (31), resulting in new dentin formation (17). Our results replicated these findings because dentin conditioning with 6% NaOCl completely abolished DSPP expression. Also consistent with previous findings, our irrigation with 17% EDTA before seeding SCAPs in scaffold promoted DSPP expression (increased by 120%). In addition, this study expanded the knowledge on the effect of dentin conditioning with NaOCl on odontoblastic differentiation by evaluating different concentrations of NaOCl. We found that the profound detrimental effect of NaOCl on DSPP expression was attenuated when lower concentrations were used. In particular, the concentration of 1.5% NaOCl had a neutral effect on DSPP expression. Moreover, the addition of a final treatment (irrigation) with 17% EDTA resulted in an increased expression of DSPP in the group treated with 1.5% NaOCl, and complete and partial reversal of DSPP-reduced expression in the groups treated with 3% and 6% NaOCl, respectively. Thus, dentin conditioning with NaOCl has a profound effect on DSPP expression, a marker of odontoblastic differentiation, with the concentration of 1.5% being the most conducive for DSPP expression.

Important biologically active growth factors are trapped in the dentin matrix during dentinogenesis. Some of these growth factors such as vascular endothelial growth factor (32) and transforming growth factor-beta 1 (33) are known to have a robust effect on the differentiation and/or proliferation of MSCs. These growth factors appear particularly efficacious in promoting the proliferation of MSCs and directing them toward an odontoblast-like phenotype (34, 35). Irrigants, especially NaOCl in high concentrations, are known to denature these dentinderived growth factors (36). In an in vivo study, DPSCs proliferated at higher rates and expressed higher levels of odontoblast-like markers in a tooth slice model compared with DPSCs placed in scaffold only (37). These findings suggest that morphogens, such as the many growth factors known to be present in dentin, are sufficient to promote the survival, proliferation, and, importantly, the differentiation of dental stem cells. EDTA is known to solubilize these growth factors from dentin, thereby increasing their bioavailability (36). Thus, in addition to removing the smear layer, providing disinfection, and possibly aiding in MSC attachment, the use of EDTA may allow clinicians the ability to harness the inductive properties of dentin-derived morphogens and growth factors normally present in dentin (38).

Several regenerative endodontic case reports have been published with great variation in treatment protocol and outcomes (1, 28). Of these, many were treated with 1.5%, 3%, or 6% NaOCl (1). Unfortunately, the high level of heterogeneity in these cases does not allow for direct comparison in clinical outcome in cases treated with various concentrations of NaOCl. Nonetheless, there has been an increase in published case reports of failed REPs that were examined histologically (39-41). The presence of cementum-like mineralization on the dentinal walls with the absence of odontoblast-like cells, except in 1 case previously diagnosed with a vital pulp (40), highlights the lack of control of the fate of stem cells in the current protocols. Although there have been reports of desirable clinical outcomes the re-establishment of a repaired tissue that resembles the undamaged pulp is desirable and must remain the goal of regenerative endodontics. Importantly, the concentrations of NaOCl tested in this study are adequate as a disinfectant and, thus, do not depart from their primary function in root canal debridement. Nonetheless, it is important to realize that current knowledge and practices must be adapted for these developing regenerative procedures for clinicians to combine the principles of disinfection and bioengineering when selecting the disinfection regimen to be used. Emerging knowledge on the various factors that affect stem cell biology may prove useful to make these procedures more predictable in order to provide maximum benefit to the patients.

Conclusions

Collectively, these results suggest that dentin conditioning with 1.5% NaOCl promoted greater survival and differentiation of SCAPs than 3% NaOCl when followed by 17% EDTA. These results contrast the lack of survival and differentiation observed when dentin was treated with 6% NaOCl, a commonly used concentration. These findings highlight that even commonly clinically used endodontic irrigants have a profound effect on stem cell survival and differentiation capacity. Thus, besides adequate disinfection, it is crucial to create a microenvironment in root canals that will promote the survival/proliferation and the differentiation of stem cells.

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

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Basic Research—Biology

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