The Effects of Irrigants on the Survival of Human Stem Cells of the Apical Papilla, Including Endocyn

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Abstract

Introduction: Endocyn, a pH-neutral solution of hypochlorous acid and hypochlorite has been developed for use as an endodontic irrigant. The purpose of this study was to evaluate the effect of Endocyn on human periodontal ligament (PDL) fibroblasts, rat osteosarcoma cells (UMR-106), and stem cells of the apical papilla (SCAP) compared with other commonly used endodontic irrigants. **Methods:** To determine cytotoxicity, cells were exposed to various concentrations of Endocyn, 6% sodium hypochlorite (NaOCl), 17% EDTA, and 2% chlorhexidine for 10 minutes, 1 hour, or 24 hours. Cell survival was measured fluorescently using calcein AM. Endocyn also was tested for its ability to inhibit SCAP proliferation and alkaline phosphatase activity. Finally, SCAP transcript expression was examined via reverse-transcriptase polymerase chain reaction. **Results:** Endocyn was no more toxic to PDL and UMR cells than water for up to 24 hours. Endocyn concentrations of 50% were toxic to SCAP after 1 hour of exposure. Endocyn concentrations of >20% inhibited SCAP proliferation, whereas concentrations of ≥10% inhibited alkaline phosphatase activity. Exposure of SCAP to 10% Endocyn for 3 days did not alter most transcript expression, but did significantly reduce the expression of alkaline phosphatase, fibromodulin, and osteomodulin. **Conclusion:** Endocyn was significantly less cytotoxic to PDL, UMR-106, and SCAP cells compared with other commonly used endodontic irrigants. High concentrations of Endocyn did inhibit some transcript expression and alkaline phosphatase activity, indicating a potential reduction in the osteogenic potential of stems cells exposed to Endocyn. *(J Endod 2017;■:1–6)*

Key Words

Cell survival, chlorohexidine, hypochlorous acid, stem cells, sodium hypochlorite

Significance

Regenerative pulp therapy requires host stem cell survival in the root canal. Most endodontic irrigants are highly cytotoxic. Endocyn is less cytotoxic to stem cells of the apical papilla and is better suited for irrigation of teeth with open apexes.

It is well established that microorganisms are the principal cause of endodontic disease (1–3), thus the paramount goal of endodontic treatment is their elimination. Regenerative endodontics is indicated for management of immature permanent teeth with pulpal necrosis. This procedure is performed with the goal of allowing and promoting further root development. This is accomplished by first disinfecting the canal space, and then allowing the ingress of stem cells into that space. Those stems cells of the apical papilla (SCAP) typically reside in close proximity to the root end and have exhibited faster proliferation, and greater differentiation into odontoblast-like cells than other dental stem cells (4, 5). SCAP survival during the disinfection process is essential to the regenerative process (6). If these stem cells are exposed to standard irrigants or medicaments, it may render them nonviable, less functional, or unable to migrate appropriately to the needed site (7, 8). This creates a conundrum regarding one's ability to adequately disinfect the canal space while promoting the survival of native cells, allowing regeneration to proceed. Sodium hypochlorite (NaOCl) has proven to be an effective irrigant because of its ability to effectively disrupt microbial biofilms (9) and dissolve necrotic tissue (10). However, a significant disadvantage of using NaOCl is its toxicity to periodontal ligament (PDL) cells (11) and SCAPs, (7) and the significant pain and morbidity involved when it is extruded beyond the confines of the tooth (12). Chlorhexidine (CHX) is another endodontic irrigant that efficiently eliminates microorganisms but also negatively impacts PDL (11) and gingival fibroblasts (13). An optimal irrigant for regenerative procedures would disinfect the canal space while not harming human cells in the vicinity (6). It has been shown that 17% EDTA promotes SCAP survival and attachment. In contrast, 2% CHX is detrimental to SCAP survival, and 6% NaOCl + EDTA decreased cell viability when compared with EDTA alone (7). Unfortunately, the use of EDTA alone is not sufficient to consistently produce a disinfected canal (14). Other intracanal medicaments, such as triple- and double-antibiotic pastes, were both detrimental to SCAP survival, whereas $Ca(OH)_2$ promoted SCAP survival and proliferation (8, 15). Thus, many root canal medicaments have been demonstrated to be detrimental to SCAP and are therefore not suitable for regenerative endodontics. The ideal endodontic irrigant would eliminate microorganisms but have insignificant or no toxicity to healthy host cells (16).

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Endocyn is a super-oxidized solution, manufactured from pure water and sodium chloride, shown to have antimicrobial properties (17, 18). According to the manufacturer, through a proprietary process, water and saltwater molecules are dissociated forming hypochlorous acid and hypochlorite ions (19). Microcyn, which is chemically similar to Endocyn, has been used as an antimicrobial wound treatment and surface disinfectant with a wide antimicrobial range in a hospital setting (18). Microcyn also has been shown to have antimicrobial properties, while also being nontoxic to human dermal fibroblast cultures when compared with hydrogen peroxide (20). It is also reported to be an effective treatment for mildly infected diabetic foot ulcers, with minimal adverse effects (21). Both Microcyn and Endocyn contain water, hypochlorous acid, and sodium chloride. However, Microcyn also contains NaOCl, whereas Endocyn also contains sodium sulfate and monobasic sodium phosphate. Thus, these solutions are similar, but not identical. Because Microcyn has shown to be a useful disinfectant while exhibiting minimal effects on human cells, Endocyn could potentially become a beneficial treatment in pulpal regeneration. The purpose of this study was to compare cell survival of PDL, osteosarcoma (UMR), and SCAP cells following exposure to Endocyn versus other traditional endodontic irrigants. This study also examined the effect of Endocyn on SCAP proliferation and gene expression.

Material and Methods

The SCAPs were obtained from Dr. Anibal Diogenes, Department of Endodontics, University of Texas Health Science Center at San Antonio. This SCAP cell line, RP-89, was derived from the apical papillae of a mandibular third molar from a single donor. RP-89 has shown to preferentially express and maintain mesenchymal stem cell markers in culture, coexpressing CD73, CD90, and CD105 in all passages evaluated. (22). Expression of these molecular markers is considered the minimal criteria for the identification of true mesenchymal stem cells (23). Cells were maintained in minimum essential medium alpha (MEMα) containing 10% fetal bovine serum (FBS) at 37°C and 5% CO_2 (Invitrogen, GIBCO, Grand Island, NY).

Human PDL fibroblasts were obtained from patients with healthy gingiva and no periodontal disease who underwent extraction at the Department of Oral Surgery at the Louisiana State University School of Dentistry, New Orleans, LA (24). All tissues were obtained from subjects after informed written consent as prescribed in an approved institutional review board protocol (#004826). Cells were maintained in MEMα containing 10% FBS at 37°C and 5% CO_2. Cell lines of rat osteosarcoma cells (UMR-106) were obtained from American Type Culture Collection (ATCC-CRL-1661; Manassas, VA) and were maintained in Dulbecco's Modified Eagle's Medium containing 10% fetal calf serum and 200 U/mL penicillin and 200 mg/mL streptomycin (Invitrogen, GIBCO). In all cases, cells were removed from polystyrene substrates using 0.25% trypsin before use in subsequent experiments. Cells were centrifuged at 200*g* for 10 minutes, resuspended in media containing 10% serum, and cell numbers verified by hemocytometer.

Irrigants

Endocyn was obtained from Sonoma Pharmaceuticals, Inc (formerly Oculus Innovative Sciences), Petaluma, CA. Sodium hypochlorite (6% solution) and CHX (Vista-CHX Plus, 480) were purchased from Inter-Med, Inc/Vista Dental Products, Racine, WI. ETDA (17% solution) was purchased from Coltene/Whaledent, Inc, Cuyahoga Falls, OH.

Exposure to Irrigants

Cells were allowed to adhere for 24 hours to 48-well plates in MEMα containing 10% FBS. The cell media was then replaced with fresh MEMα containing various dilutions of endodontic irrigant obtained by diluting 6% NaOCl, distilled water, 2% CHX, 17% EDTA, and Endocyn. MEMα alone served as a negative control. Following exposure to the solutions for 10 minutes, 1 hour, or 24 hours, the cells were treated with calcein AM for 1 hour to render live cells fluorescent. Cells were rinsed twice with phosphate-buffered saline (PBS) and cell viability was determined using a Synergy 2 plate reader (Biotek Instruments, Inc, Winooski, VT), calibrated to measure fluorescence intensity with filters appropriate for approximately 480-nm excitation and approximately 520-nm emission. Values represent the means and standard deviations (SDs) of 8 samples (after subtraction of autofluorescence).

Cell Proliferation

SCAP cells were plated into 48-well tissue culture plate at 4000 cells in 200 μL in MEMα, and allowed to adhere for 1 hour. Cells were incubated for up to 9 days at 37°C and adherent cells were rinsed with PBS and quantified fluorometrically using CyQuant fluorescent dye (Molecular Probes, Eugene, OR) (25). Fluorescence was measured using a Synergy 2 plate reader (Biotek Instruments, Inc) with filters appropriate for approximately 480-nm excitation and approximately 520-nm emission. Values represent the means and SDs of 8 samples (after subtraction of autofluorescence).

Alkaline Phosphatase Activity

SCAP cells exposed to Endocyn were assayed for alkaline phosphatase activity (25). Cells were plated into 48-well plates and exposed to various concentrations of Endocyn for up to 9 days. Cells were rinsed 3 times in PBS and frozen to lyse cells. P-nitrophenol phosphate 1 mg/mL in 0.1 M diethanolamine (pH 8.3) was added to each well (200 μL per well) and incubated at 25°C for 30 minutes with gentle agitation. The enzymatic color reaction was stopped by the addition of 500 μL 0.75 N NaOH, and assayed for 405-nm absorbance in a Biotek Synergy 2 plate reader (Biotek Instruments, Inc). Values represent the means and SDs of 8 samples.

Comparison of Transcript Expression

Differential transcript expression was examined by reverse-transcriptase polymerase chain reaction (PCR). Briefly, RNA was extracted from tissue and cell samples using Qiagen kits (Germantown, MD) and gene expression determined by reverse-transcriptase PCR. The number of cycles was chosen by creating a standard curve using serial dilutions of DNA template known to include the genes in question, and determining the PCR conditions that reveal twofold differences in gene expression over 3 orders of magnitude. Twenty-five to 35 cycles of PCR were chosen to be within the linear range of detection for all of the genes examined. The primers for these studies were derived from the published DNA sequences of human genes as described previously (24, 26).

Statistical Analysis

For quantitative analysis, 8 samples were averaged and the means and SDs were compared with the control values for untreated cells. Comparisons of experimental groups were performed via analysis of variance where normality of the data was confirmed using the Shapiro-Wilk normality test, with $P < .05$ considered to be significant.