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Effect of sodium hypochlorite on human pulp cells: an in vitro study

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Abstract

Background—The purpose of this study was to determine the effect of sodium hypochlorite (NaOCl) on human pulp cells to provide an aid in determining its optimum concentration in maintaining the viability of remaining pulp cells in the revascularization of immature permanent teeth with apical periodontitis.

Study design—Human pulp tissue cells taken from extracted third molars were plated, incubated, and subjected to various concentrations of NaOCl (0.33%, 0.16%, 0.08%, and 0.04%) for 5-, 10-, and 15-minute time intervals to simulate possible contact times in vivo. The Cell Titer–Glo Luminescent Cell Viability Assay was used to determine the number of viable cells present in culture following treatment.

Results—The results showed an increase in cell viability with the lowering of NaOCl concentration. The use of 0.04% NaOCl was similar to the control, indicating nearly complete preservation of cell viability at all time intervals tested. As sodium hypochlorite concentration increased from 0.04% to 0.33%, cell viability decreased correspondingly.

Conclusions—The results indicate that the lowest concentration of NaOCl tested did not affect the viability of cells. This may prove beneficial in developing a new treatment protocol to help preserve existing vital pulp cells in revascularization cases.

INTRODUCTION

Sodium hypochlorite is routinely used in nonsurgical endodontic therapy as an effective antimicrobial agent for its chemical dissolution properties and as a lubricant during instrumentation.¹ Sodium hypochlorite is also routinely used in the revascularization process to help reestablish the vitality in a nonvital tooth to allow repair and regeneration of the tissues.² Nygaard-Ostby and Hjortdal began studying pulp regeneration in the early sixties.³ Since then, there has been a recent resurgence of interest relating to revascularization treatment protocols. Because mechanical instrumentation should be minimized or eliminated in revascularization cases to help prevent the weakening of already thinned dentinal walls,

there is a larger reliance on irrigation and interappointment antibiotics in providing an environment conducive to normal root development.⁴⁻⁶

It has been speculated that vital pulp tissue remnants and Hertwig epithelial root sheath play important roles in the successes we have seen thus far in revascularization cases.⁶ After canal disinfection and a reduction in the inflammatory process, the remaining vital pulp tissue remnants may proliferate, allowing for continued root development.⁶⁻⁷ It is of critical importance that clinicians select irrigants and medicaments that minimize harm to remaining vital pulp cells, contributing to the “regeneration of a functional pulp-dentin complex.”⁸ We should redirect our focus toward using concentrations in our irrigating solutions that promote maximum pulp cell viability.

Current treatment protocols advocate the use of full strength sodium hypochlorite along with a triple antibiotic paste (a combination of ciprofloxacin, metronidazole, and minocycline) in the revascularization process.^{6, 8-9} A clinical report showed successful revascularization treatment on a mandibular second premolar with apical periodontitis.⁶ As part of the treatment protocol, full-strength sodium hypochlorite was used at both first and second appointments.

In a review of past research, it seems that using a reduced concentration of sodium hypochlorite in the revascularization process may help to preserve any remaining vital pulp cells while maintaining desirable antibacterial properties. No significant difference in antibacterial effect was noticed by using either 0.5% or 5% sodium hypochlorite.¹⁰ Baumgartner and Cuenin¹¹ found no significant difference in the ability of varying concentrations of sodium hypochlorite to remove pulpal remnants and predentin in the middle third of root canals. Similarly, Jung et al.⁴ presented a case series where they also performed revascularization on permanent teeth with pulpal necrosis. They used full-strength sodium hypochlorite in one-half of their cases and half-strength sodium hypochlorite in the remainder. Interestingly, they achieved long-term clinical success regardless of the concentration of sodium hypochlorite used to treat the root canal space.

In light of these findings, the use of more dilute concentrations of sodium hypochlorite, such as that of Dakin solution, may provide an efficacious and more biologically compatible alternative.^{12, 13} Not only would it be desirable to use an irrigant that was antimicrobial and less cytotoxic to remaining vital pulp cells, it would also be beneficial to use an irrigant that is kinder to the periapical tissues in cases of inadvertent extrusion beyond the periapex. The purpose of the present study was to determine the effect of sodium hypochlorite on human pulp cells to provide an aid in determining its optimum concentration as an irrigant for use in the revascularization of immature permanent teeth with apical periodontitis.

MATERIAL AND METHODS

Culture of primary human pulp cells

The primary human pulp cells used in this study were established from pulp tissue obtained from noninfected third molars. Teeth from individuals who underwent extraction at the Department of Oral and Maxillofacial Surgery at the University of Alabama at Birmingham

were used with Institutional Review Board approval. The pulp cells were cultured in Alpha Minimum Essential Medium (α MEM). The media was supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin. Cells were grown at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Cell growth was monitored with a Nikon Eclipse 80i upright fluorescence microscope. Once the plated human pulp cells reached confluency after day 6, they were passed into 3 12-well experimental plates. To do this, the medium was aspirated from the 10 cm plate and the cells rinsed with 10 mL phosphate-buffered saline solution (PBS). For cell detachment, 1.5 mL trypsin-EDTA (0.25%) was overlaid onto the washed cells. To achieve efficient digestion, the plate was then placed in the incubator at 37°C for 2–4 minutes. Care was taken not to overdigest the cells with the trypsin-EDTA. To stop the digestion reaction and to collect the cells, 5 mL α MEM was added to the plate. With a micropipette, medium was pipetted up and down to facilitate the detachment of the pulp cells from the plate and to break up clumps of cells. The collected cells were then diluted with 6.5 mL medium for counting with a hemocytometer. For plating, pulp cells (3×10^6) were diluted in 36 mL α MEM and thoroughly mixed. A total of 1 mL cell slurry was added into each well of 3 12-well plates. The remaining cells were plated on to a 10-cm plate for carrying the culture. All plates were placed in an incubator maintained at 37°C with 5% CO₂ and 95% air.

Determination of nontoxic dose of sodium hypochlorite

To determine a possible range of NaOCl concentrations and the cytotoxicity effects, a pilot experiment was conducted. The pilot study was carried out to determine the nontoxic dose of sodium hypochlorite on human pulp cells in the same way as the actual study. NaOCl concentrations of 0.33%, 0.16%, 0.08%, and 0.04% were used. To prepare these serial dilutions, the following formula was used:

$$(\text{concentration desired}/\text{NaOCl concentration}) \times \text{volume needed}$$

For obtaining the desired concentrations in 1 mL α MEM, 62.8, 30.5, 15.2, and 7.6 μ L of NaOCl were added. The total volume was made up to 1 mL with the addition of 937, 969, 984, and 992 μ L of growth media, respectively. Physiologic saline buffer was used as the control.

Human pulp cells were exposed to each concentration of NaOCl for 5, 10, and 15 minutes. These time intervals were selected to reflect possible clinical contact times. For accuracy and reproducibility of results, each treatment was done in triplicate. On reaching confluency (48 hours after plating), each plate was removed from the incubator as needed. A micropipette was used to deliver the desired concentration of NaOCl to each well for the specific time periods of 5, 10, and 15 minutes. Once the desired contact time for each concentration of NaOCl was reached, the combination of NaOCl and growth medium was aspirated from each experimental well and replaced with 1 mL new growth medium to halt the reactions. In this pilot experiment, 3 independent wells were used for each concentration. Plates were then placed in the incubator at 37°C for a 24-hour incubation period and observed under a light microscope to see how the human pulp cells reacted to the varying concentrations of NaOCl. Figure 1 is a visual representation of our findings. From the

results of this pilot study, we decided to use the same NaOCl concentrations in the final experiment.

Cell viability assay

After the passage, 3 12-well plates were seeded with human pulp cells. Upon reaching confluence, procedure of the final experiment was carried out in the same manner as described above. After aspiration of the varying concentrations of NaOCl and growth media for the specific time intervals of 5, 10, and 15 minutes, 1 mL of fresh growth medium was added to each of the experimental wells to stop the reactions. The plates were returned to the incubator at 37°C for a 24-hour period. Pictures were taken of each group of cells with a digital epifluorescence microscope. Pulp cell viability was assessed with the Cell Titer–Glo Luminescent Cell Viability Assay (Promega Corp., Madison, WI). This assay was used to determine the number of viable cells in culture based on quantification of the adenosine triphosphate (ATP) present, which signals the presence of metabolically active cells. One day later, morphology of treated pulp cells was documented and 500 µL of new growth medium was added to each well. The plates were allowed to equilibrate to room temperature (22°C) for 30 minutes. Equal amounts of the Cell Titer–Glo Substrate and the Cell Titer–Glo Buffer (500 µL) were mixed and added to each of the wells containing 500 µL growth medium to provide a combined total volume of 1 mL. To initiate cell lysis, plates were placed on an orbital shaker for 2 minutes. Lysis reactions were carried for 10 minutes. The 250 µL of cell lysates from each well was transferred to clear plastic tubes and the ATP measured with the FB12 Luminometer Version 2.0. A total of 36 different luminescence readings (3 time intervals in triplicate) were taken for analysis.

RESULTS

Morphology and growth profile of primary human pulp cells

Human pulp cells from extracted third molars were cultured to determine the effect of sodium hypochlorite. Cells began to adhere to the plates within 4–6 hours. Most of the cells were attached to the plate by 24 hours, and they changed morphologically from a rounded shape to one that was more flattened and elongated. As the cells became flattened, they underwent duplication and doubled their number by day 3. Full confluency was achieved by day 6, as reflected by the tightly packed cells.

Assessment of optimal sodium hypochlorite concentration for viability of pulp cells

Human pulp cells were plated, incubated to confluency, and subjected to varying concentrations of NaOCl. As a control, cells were exposed to physiologic saline buffer. Effects of NaOCl on cell morphology, cell proliferation, and cell survival were documented 24 hours after treatment (Fig. 1). Normal morphology of actively dividing pulp cells is evident in control cells (0.00) exposed to PBS for 5, 10, and 15 minutes. When cells were exposed to low concentrations of NaOCl (0.04% and 0.08%), no significant differences in morphology or proliferative capacity was noted. In sharp contrast, higher concentrations of NaOCl (0.16% and 0.33%) caused significant changes in cell morphology and cell number even when the exposure time was 5 or 10 minutes. Massive cell death was noted when cells

were treated with 0.33% dilution of NaOCl for 15 minutes. These dilutions and exposure times were selected to simulate clinical contact times.

Maximum viability of pulp cells is achieved with a 76% dilution of full-strength NaOCl

Maintenance of viable pulp cells is essential for the revascularization process of immature permanent teeth with apical periodontitis. We examined the optimal concentration of NaOCl that results in maximal viability of pulp cells. The Cell Titer–Glo Luminescent Cell Viability Assay was used to determine cell viability. This assay lyses cells, causing a luminescent signal proportional to the amount of ATP present, which, in essence, is a measure of cell viability. Cell viability was measured in relative luminescent units (RLU). We found a time- and NaOCl concentration–dependent effect on pulp cell viability. Exposure to 0.04% NaOCl did not cause any noticeable change in pulp cell viability, which was very similar to untreated control cells. In sharp contrast, a 30-fold decrease in pulp cell viability was noted with the 0.08% NaOCl concentration at the 5-minute contact time (18 million RLU to 0.6 million RLU). The cell viability was further decreased 1.5- and 2-fold with 10 and 15 minute exposure times, respectively. Surprisingly, no change in cell morphology was evident at this concentration. Use of higher concentrations (0.16% and 0.33%) resulted in a 90- and 180-fold decrease in cell viability compared with control cultures (18 million RLU to 0.01 million RLU). These changes were coupled with a severe alteration in cellular morphology and complete cell death within 24 hours.

DISCUSSION

Several case reports concerning the revascularization of immature permanent teeth with apical periodontitis have been presented.^{2, 4, 6–7} Although many of these case reports and studies have shown promising results, revascularization treatment protocols vary widely. The present study evaluated the effect of NaOCl concentration on human pulp cells at different time intervals.

Our study showed that cultured human pulp cells exhibited the greatest viability when subjected to the lowest concentration (0.04%) of NaOCl tested. Current protocols for the revascularization of immature permanent teeth with apical periodontitis typically advocate the use of half-strength (2.5%) or full-strength (5.25%) NaOCl for irrigation at the first and second appointments. Our results suggest a potential increase in successful treatment outcomes by maintaining viability of the existing pulp cell population when using even lower concentrations of NaOCl.

As clinicians, we may be decreasing our incidence of clinical success by using the currently advocated NaOCl concentrations in revascularization cases. An earlier report showed a statistically significant reduction in cultured bacteria samples taken from immature infected dog teeth with apical periodontitis after a 2-week treatment with a triple antibiotic paste consisting of ciprofloxacin, minocycline, and metronidazole.¹⁴ After initial irrigation of each canal with 10 mL of 1.25% NaOCl, only 10% of the canals cultured bacteria free. However, when followed up with the 2-week triple antibiotic paste, 90% of the canals cultured bacteria free. This result sheds light on the fact that much of the bacterial disinfection lies in the administration of the triple antibiotic paste in addition to NaOCl

irrigation. Furthermore, if the triple antibiotic paste is used as an interappointment medicament, greater increases in root length are achieved.^{9, 15} Because the results of our study showed that a 0.04% NaOCl concentration led to nearly complete pulp cell viability in vitro, one must consider the possible benefit of using a more dilute concentration of NaOCl, especially during the second visit of revascularization cases to promote the viability of remaining pulp cells.

Because the necessary antimicrobial activity and tissue dissolution are achieved during the first appointment of revascularization procedures by using full- or half-strength NaOCl along with a triple antibiotic paste, it seems logical to use a more dilute form of NaOCl at the second appointment to maintain and encourage viable pulp cells. This decreased concentration should promote an environment more conducive to cell proliferation. In a recent study, Lovelace et al.¹⁶ found saline solution as the sole irrigant at the second appointment to be effective for pulp regeneration. A dilute concentration of NaOCl, such as the 0.04% NaOCl used in the present study, while still having significant antimicrobial properties, should help to promote cell viability.

We think that maintaining the existing vital pulp cell population in revascularization cases would play a major role in promoting successful treatment outcomes. Because it has been shown that the triple antibiotic paste used in many revascularization studies^{4–8, 15} has shown excellent antimicrobial efficacy and is “well tolerated by vital pulp tissue,”^{14, 17} using higher concentrations of NaOCl, especially at the second appointment, may be more detrimental to the existing vital pulp cell population. In addition, using lower NaOCl concentrations would reduce its cytotoxic effect in cases of inadvertent extrusion beyond the apical foramen.¹⁸

Continuation of this research might be to take the more dilute 0.04% NaOCl concentration, which promoted the greatest cell viability in this study, and implement it during the second appointment of revascularization treatment protocols in humans and animals to shed light on its possible significance in vivo. Furthermore, a similarly diluted NaOCl concentration tested by Omidbakhsh¹⁹ showed significant antimicrobial effectiveness. This change in treatment protocol may lead to increased clinical success in the revascularization of immature permanent teeth with apical periodontitis.

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Figure 1. Decreased cell viability and altered morphology of pulp cells exposed to sodium hypochlorite

Primary human pulp cells were seeded in 12-well culture dishes and exposed to the indicated concentrations of NaOCl for 5, 10, and 15 minutes. Pictures were taken 24 hours later, and a representative image of each treatment group performed in triplicate is shown (top). Note the change in cell density and morphology with higher NaOCl concentration. Pulp cells died when exposed to 0.33% NaOCl for 10 and 15 minutes (rounded floating cells). Cell viability was determined with the Cell Titer-Glo Luminescent Cell Viability Assay as described in the Materials and Methods section.