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## The effect of sodium hypochlorite and chlorhexidine on cultured human periodontal ligament cells

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**Objective.** The objective of this study was to examine the effects of sodium hypochlorite (NaOCl) and chlorhexidine (CHx) on cultured human periodontal ligament (PDL) cells in vitro.

**Study design.** The effects of irrigation solutions on human PDL cells were evaluated by propidium iodide fluorescence cytotoxicity assay, protein synthesis assay, and mitochondrial activity.

**Results.** Both NaOCl and CHx were cytotoxic to human PDL cells in a concentration- and contact time-dependent manner. In addition, CHx inhibited protein synthesis in human PDL cells. Although NaOCl displayed cellular cytotoxicity, it showed no protein inhibition in the PDL cells. Furthermore, both NaOCl and CHx exhibited an inhibitory effect on mitochondrial activity on human PDL cells.

**Conclusions.** This study suggests that these irrigation fluids may cause detrimental effects on vital tissues. Its clinical significance, however, needs to be evaluated further because concentration used, exposure time to the agent, and exposure surface area are important factors affecting the resulting effect.

(*Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2001;92:446-50)

Irrigation solutions are used in endodontic therapy to remove debris from the root canal, eliminate microorganisms, and serve as a lubricant during instrumentation. Therefore, an "ideal" irrigation solution should be efficient as an antimicrobial agent but not toxic to the periodontal tissues.

Sodium hypochlorite (NaOCl) and chlorhexidine (CHx) have been used for root canal irrigation in endodontic therapy. NaOCl has been widely recommended as an irrigation solution to aid in the chemomechanical debridement of the root canal system because of its dissolving action on pulp tissue<sup>1,2</sup> and its antimicrobial properties.<sup>3,4</sup> Because of its substantive antimicrobial properties, CHx has become an effective oral antimicrobial agent for use in periodontal therapy and caries prevention and a therapeutic agent for other oral infections.<sup>5,6</sup> Recent studies using CHx as an endodontic irrigating solution have shown its antimicrobial efficiency and extended residual activity.<sup>7-9</sup>

During endodontic treatment the irrigating solution will be in contact with pulpal and periapical tissues. Debris as well as irrigating solutions may also be

pushed beyond the apical foramen and cause further periapical complications.

The purpose of this study was to elucidate the potential toxicologic implications of NaOCl and CHx on periapical periodontal tissues. Because the ultimate goal in endodontic therapy is the healing and regeneration of periapical tissues, the objective was to evaluate the cytotoxicity of various concentrations of the irrigation solutions on cultured human periodontal ligament (PDL) cells because these cells are responsible for normal maintenance and the regeneration of the periodontium.<sup>10</sup>

### MATERIAL AND METHODS

All tissue culture biologicals were purchased from Gibco Laboratories (Grand Island, NY). NaOCl (5.25%) was obtained from Shimakyu's Pure Chemicals (Osaka, Japan). CHx was prepared from Hibitane (5% W/V chlorhexidine gluconate; Zeneca Limited, Macclesfield, Cheshire, UK). Propidium iodide (PI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and dimethyl sulfoxide were purchased from Sigma Chemical Co (St Louis, Mo). [<sup>3</sup>H]-leucine was obtained from Amersham International PLC (Amersham, Buckinghamshire, UK). Both NaOCl and CHx were dissolved in the culture medium immediately before each experiment.

### Cell culture

Human PDL cells were cultured by using an explant technique described previously.<sup>11,12</sup> Human PDL cells were cultured from the roots of premolars extracted for orthodontic reasons. After extraction, teeth were rinsed

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Received for publication Nov 8, 2000; returned for revision Jan 3, 2001; accepted for publication Apr 9, 2001.

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1079-2104/2001/\$35.00 + 0 7/15/116812

doi:10.1067/moe.2001.116812

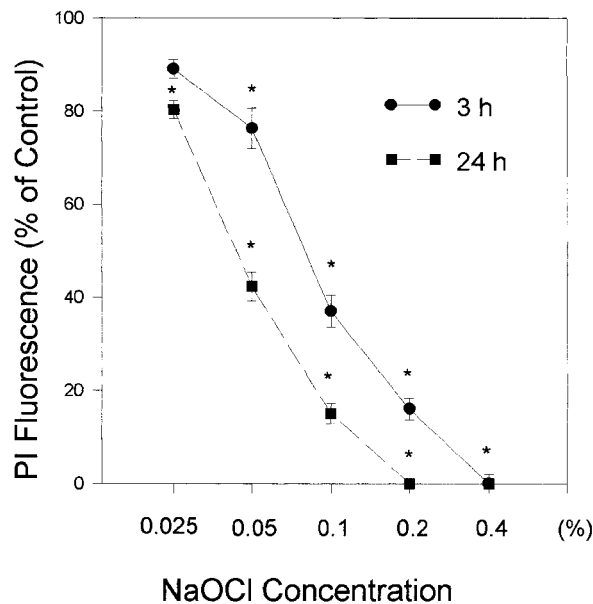


Fig 1. Effects of NaOCl on human PDL cells by PI fluorescence assay for 3-hour and 24-hour incubation periods. Percentage of fluorescence at each concentration compared with that of control was calculated. Each point and bar represent a mean  $\pm$  SD. Asterisk denotes significant differences from control value, with  $P < .05$ .

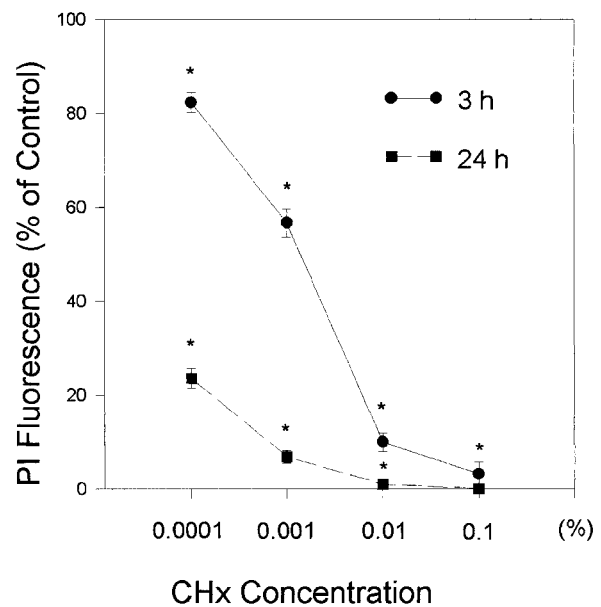


Fig 2. Effects of CHx on human PDL cells by PI fluorescence assay for 3-hour and 24-hour incubation periods. Percentage of fluorescence at each concentration compared with that of control was calculated. Each point and bar represent a mean  $\pm$  SD. Asterisk denotes significant differences from control value, with  $P < .05$ .

with Hanks' buffered saline solution and then placed in 60-mm Petri dishes containing Dulbecco's modified Eagle's medium (DMEM) and 100 units of penicillin and 100  $\mu$ g of streptomycin/mL. To avoid contamination from the gingiva, the periodontal ligament was carefully removed from the middle third of the root by scalpel. The fragments were grown in DMEM supplemented with 10% fetal calf serum (FCS) and antibiotics. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. Experiments with PDL fibroblasts were conducted by using cells between the third and eighth passage.

### Cytotoxicity assay

Cytotoxicity was assessed by PI fluorescence cytotoxicity assay according to a method of Skehan et al,<sup>13</sup> but modified slightly.<sup>14,15</sup> PI is a general biomass stain that binds to double-stranded nucleic acids (RNA and DNA) and results in an enhancement of PI fluorescence.<sup>16</sup> Briefly, fibroblasts were seeded by  $5 \times 10^4$  cells per well in 24-well culture plates and incubated for 24 hours. After overnight attachment, the culture medium was replaced with fresh DMEM containing 5% FCS and the irrigation solutions. The final concentrations used were 0.025%, 0.05%, 0.1%, 0.2%, and 0.4% of NaOCl and 0.0001%, 0.001%, 0.01%, and 0.1% of CHx. Three or 24 hours later, plates were harvested by

first freezing at -20°C for 2 hours and then thawing at 50°C for 15 minutes. After the freeze-thaw treatment, 200  $\mu$ g/mL of PI was added to each well. After 1-hour incubation, the PI fluorescence was read in a fluorescence measurement system (CytoFluor 2300; Millipore, Bedford, Mass, USA) at wavelengths of 531/604 nm.

### Protein synthesis

Protein synthesis was measured in 24-well culture plates. Each well was incubated with  $5 \times 10^4$  cells in 1 mL of DMEM with 10% FCS and incubated overnight. The medium was replaced with 1 mL fresh medium containing 2% FCS and CHx in a final concentration of 0.001%, 0.005%, 0.01%, 0.05%, and 0.1%. The rate of protein synthesis was estimated by the incorporation of [<sup>3</sup>H]-leucine. After labeling with tritiated leucine for 2 hours, the radioactive medium was decanted. Monolayer cells were removed by trypsinization (0.025%) and isolated by centrifugation, and cellular proteins were precipitated with 5 mL of cold 5% trichloroacetic acid. The final precipitates were each dissolved in 0.5 mL 2 mol/L NH<sub>4</sub>OH and transferred to scintillation vial inserts containing 3 mL of scintillation cocktail. Radioactivity was measured in a liquid scintillation counter (Packard model 2100TR; Packard, Downers Grove, NJ), and results were expressed as percent inhibition of leucine incorporation.

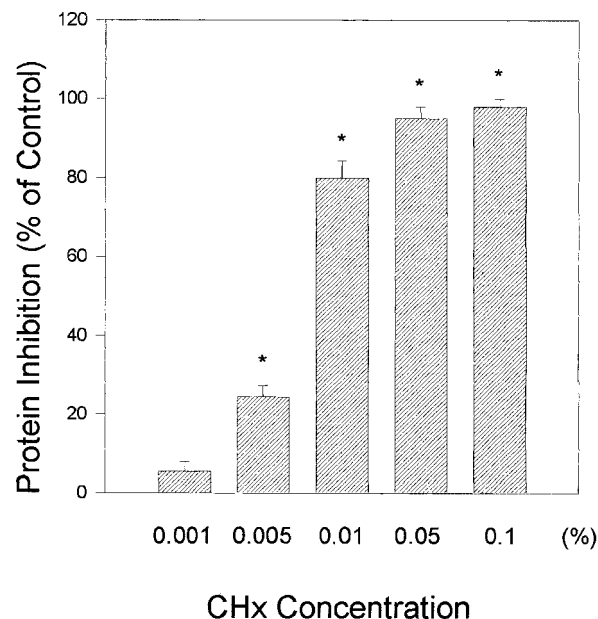


Fig 3. Effects of CHx on protein synthesis of human PDL cells during 2-hour incubation. All values are means of [ $^3\text{H}$ ]-leucine incorporation by 3 independent tests  $\pm$  SD (bars). Asterisk denotes significant differences from control values, with  $P < .05$ .

### Mitochondrial activity

Effects of irrigation solutions on the mitochondrial function were measured by a colorimetric assay as described by Mosmann.<sup>17</sup> This assay measures the conversion of a yellow water-soluble MTT dye into a purple formazan product by active mitochondria via an electron current. According to our recent study,<sup>18</sup> MTT solution was prepared in 5 mg/mL of phosphate-buffered saline just before use and filtered through a 0.22- $\mu\text{m}$  filter. PDL cells were seeded  $2 \times 10^4$  cells/well into 96-well culture plates. After overnight attachment, cell was treated with various concentrations of irrigation solutions, and 10  $\mu\text{L}$  of MTT solution was added to each well for 2 hours. The final concentration was 0.025%, 0.05%, 0.1%, 0.2%, and 0.4% for NaOCl and 0.001%, 0.005%, 0.01%, 0.05%, and 0.1% for CHx. On termination of the experiment, all the medium was discarded by inverting and tapping the plates, and 100  $\mu\text{L}$  of dimethyl sulfoxide was added to each well. The spectrophotometric absorbance at 540 nm was then measured by an enzyme-linked immunosorbent assay reader (Hitachi, U2000, Tokyo, Japan). The functional mitochondrial activity of irrigation solution-treated cells was calculated as a percentage of control.

### Statistical analysis

Five replicates of each concentration were performed in each test. All assays were repeated at least 3 times to

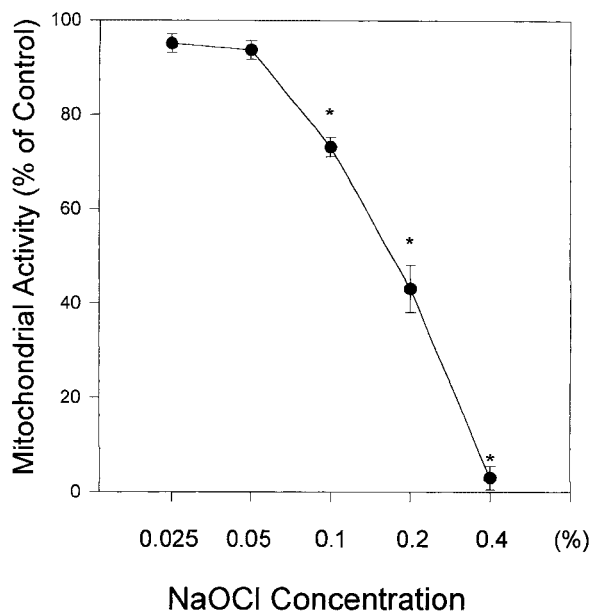


Fig 4. Effects of NaOCl on the functional mitochondrial activities by MTT assay after 2-hour incubation. Results are expressed as percentages of untreated controls. All values are shown as mean  $\pm$  SD. Asterisk denotes significant differences from control values, with  $P < .05$ .

ensure reproducibility. The significance of difference between the control and treated groups was statistically analyzed by one-way analysis of variance. Tests of differences of the treatments were analyzed by the Duncan test, and a  $P$  value less than .05 was considered statistically significant.

### RESULTS

By measuring PI fluorescence with narrow bandwidth 531/604 nm excitation/emission filters, the effects of various concentrations of irrigation fluids on double-stranded polynucleic acid contents of cultures were visualized (Figs 1 and 2). Both NaOCl and CHx were cytotoxic to human PDL cells in a concentration- and time-dependent manner ( $P < .05$ ). NaOCl was cytotoxic to PDL cells at the concentrations of 0.025% or greater (Fig 1). CHx was cytotoxic to PDL cells at the concentrations of 0.0001% or greater (Fig 2). At the same concentration of NaOCl and CHx, chlorhexidine was more cytotoxic to human PDL cells than NaOCl ( $P < .05$ ).

[ $^3\text{H}$ ]-leucine was used for monitoring protein synthesis. As shown in Fig 3, CHx inhibited protein synthesis in human PDL cells. CHx inhibited protein synthesis at 0.005% and greater concentrations in a dose-dependent manner ( $P < .05$ ). A 0.01% concentration level of CHx significantly inhibited the protein synthesis in approxi-

mately 80% of those in the untreated control group. The protein synthesis was almost completely inhibited by the concentration greater than 0.05%.

Although NaOCl displayed cellular cytotoxicity, it showed no protein inhibition in human PDL cells. No difference in the percentage of [<sup>3</sup>H]-leucine incorporation was noted in any NaOCl-affected cells.

Both NaOCl and CHx exhibited an inhibitory effect on mitochondrial activity on human PDL cells (Figs 4 and 5). At concentrations of 0.1% through 0.4%, NaOCl inhibited 27% through 97% of functional mitochondrial activities ( $P < .05$ ). A 0.001% concentration level of CHx significantly inhibited the mitochondrial activity in about 25% of those in the untreated control group. The mitochondrial activity was almost completely inhibited at a concentration of 0.125% of CHx (Fig 5).

## DISCUSSION

The toxic effects of materials used in endodontic therapy are of particular concern because damage or irritation could cause degeneration of the periapical tissue and delayed wound healing. Ideally, an endodontic irrigating solution should be selectively toxic and act as an antimicrobial agent but with low periapical tissue toxicity.

With the simple PI fluorescence assay<sup>13</sup> the cellular responses could be measured without a complicated process. The 531/604 nm wavelengths of excitation/emission combinations reflect cellular RNA more than DNA. Because RNA usually is synthesized earlier than protein in cellular growth, the PI assay is a much better tool to detect cellular responses than protein, biomass, and DNA assays.<sup>19</sup> It is usually the method of choice, when a short assay is desirable. Our previous study has shown that this assay is useful for preliminary cytotoxicity screening of root canal medicaments in vitro.<sup>15</sup> In this study, NaOCl and CHx were found to be cytotoxic to human PDL cells in a dose- and time-dependent manner by using the PI fluorescent assay.

NaOCl has been used in endodontics at different concentrations ranging from 0.5% to 5.25%. At a concentration of 0.4% of NaOCl, the cells showed no PI activity, and it can therefore be assumed that the cells were dead or dying. With this experimental technique some cytotoxic effect of NaOCl could be observed at concentrations as low as 0.01%. Our results were generally in agreement with that reported by Koulaouzidou et al.<sup>20</sup> However, our results differed from those of Spangberg et al,<sup>21</sup> who recommended 0.5% NaOCl as an acceptable noncytotoxic solution, and Hegger et al,<sup>22</sup> who reported 0.025% NaOCl to be non-tissue toxicity. The reason for this contrary result is not clear. It may result from different origins of the

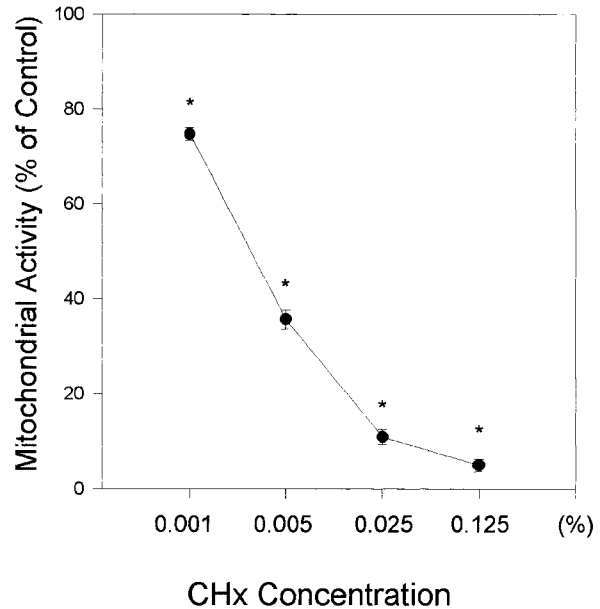


Fig 5. Effects of CHx on the functional mitochondrial activities by MTT assay after 2-hour incubation. Results are expressed as percentages of untreated controls. All values are shown as mean  $\pm$  SD. Asterisk denotes significant differences from control values, with  $P < .05$ .

cells or different experimental protocols used in each laboratory. The cellular effects of NaOCl may not necessarily be comparable in all tissues.

Although NaOCl was found to inhibit mitochondrial activity in a dose-dependent manner, no protein inhibition could be observed. From the PI fluorescence assay, the macromolecule inhibition of NaOCl might be via DNA or RNA synthesis. However, the detailed mechanism of macromolecule inhibition remains to be further defined.

CHx is a cationic bisbiguanide with excellent antimicrobial action. It is active against a wide range of microorganisms because it is bacteriostatic at low concentrations and bactericidal at higher concentrations.<sup>23</sup> CHx, however, was found to be highly cytotoxic to human PDL cells by inhibiting double-stranded nucleic acid content, protein synthesis, and mitochondrial activity. Previous studies have shown that CHx was cytotoxic to human fibroblasts via inhibition of protein synthesis.<sup>24,25</sup> The highly cationic nature of the drug may have a general inhibitory effect on the cells, or the CHx may affect protein biosynthesis specifically.

Regeneration of the lesion and the periodontal connective tissue attachment apparatus after endodontic treatment is important. Toxic antimicrobial agents used in the root canal may impede periapical tissue healing. Studies have suggested that the cell growth, proliferation, and matrix synthesis of fibroblasts are necessary

for regeneration.<sup>26,27</sup> Both NaOCl and CHx were found to impair cell function of the target cells at various concentrations. These detrimental effects of irrigation solutions might impair the reparative and regenerative potential of periapical tissues.

It has been reported that periapical tissue damage is increased in necrotic pulp cases and in teeth with root canals with large apical foramina, because materials used in the canal will more readily leak beyond the apical foramen.<sup>28</sup>

This study suggests that these irrigation fluids may cause detrimental effects on vital tissues. Its clinical significance, however, needs to be evaluated further because concentration used, exposure time to the agent, and exposure surface area are important factors affecting the resulting effect. All this must be considered when selecting the appropriate agent.

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