Chromographic Analysis and Cytotoxic Effects of Chlorhexidine and Sodium Hypochlorite Reaction Mixtures

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Abstract

Introduction: The literature reveals controversies regarding the formation of para-chloroaniline (PCA) when chlorhexidine (CHX) is mixed with sodium hypochlorite (NaOCl). This study aimed to investigate the stability of PCA in the presence of NaOCl and to examine the *in vitro* cytotoxic effects of CHX/NaOCl reaction mixtures. **Methods:** Different volumes of NaOCl were added to CHX (mix 1) or PCA (mix 2). Upon centrifugation, the supernatant and precipitate fractions collected from samples were analyzed using high-performance liquid chromatography. The cytotoxic effects of both fractions were examined on human periodontal ligament and 3T3 fibroblast cell lines. **Results:** High-performance liquid chromatographic analysis showed no PCA signal when NaOCl was mixed with CHX (mix 1). In mix 2, the intensity of PCA was decreased when NaOCl was added to PCA, and chromatographic signals, similar to that of CHX/NaOCl, were also observed. The mortality of precipitates exerted on both cell lines was lower compared with that of supernatants. **Conclusions:** The discrepancy in the data from the literature could be caused by the instability of the PCA in the presence of NaOCl. The CHX/NaOCl reaction mixture exhibits a wide range of cytotoxic effects. *(J Endod 2017;43:1545–1552)*

Key Words

Brown precipitate, chlorhexidine, cytotoxicity, para-chloroaniline, sodium hypochlorite

Significance

The NaOCl/CHX combination may lead to the formation of transient PCA and other degradative by-products, which can potentially exert toxic effects on the periapical tissues.

Endodontic failure may occur because of bacterial persistence in the root canal system as a consequence of poor disinfection and debridement of the pulp space, untreated canals, inadequate filling, or coronal leakage (1). Mechanical instrumentation alone cannot completely clean the root canal system (2). Thus, a large array of irrigating and disinfecting solutions has been used to assist in the debridement of root canals (3). Commonly used methods of disinfection in endodontic treatments rely on the use of sodium hypochlorite (NaOCl) followed by the use of other adjunct irrigants such as chlorhexidine gluconate (CHX).

NaOCl solution is used to irrigate the root canal system because of its dissolving action on pulp tissue, other organic materials (4, 5), and bactericidal properties (6, 7). Unfortunately, in some cases, NaOCl alone is not sufficient for total disinfection of the root canal system (8, 9). For this reason, other substances, such as CHX, are used after irrigation with NaOCl to improve microbicidal properties. CHX is a popular antiseptic compound that shows a potent effect against most gram-positive and some gram-negative bacteria (10, 11), fungi, and yeast (12). In general, CHX efficacy is related to its concentration and frequency of use (13). Notably, if NaOCl is still present in the canal when CHX is added, a brown precipitate is formed (3, 14), with consequent surface discoloration and a possible negative effect on the sealing ability of the obturation material and outcome of the treatment (14–16). Thus, an intermediate flush should be used after each irrigating solution followed by dryness of the canals to prevent further reactions among the components (17, 18).

Studies found that the brown precipitate contains a significant amount of para-chloroaniline (PCA) detected through X-ray photoelectron spectroscopy, time-of-flight secondary ion mass spectrometry (19), and gas chromatography–mass spectrometry (20). However, investigators (21) found that no PCA was detectable when nuclear magnetic resonance was used. The same finding was observed in a recent study when analysis was performed using high-performance liquid

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TABLE 1. Experimental Conditions Used in Cytotoxic Studies

Cell types	Specimens	Incubation time	Dilutions
3T3 or HPLFs	1 mL 2.0% CHX + 1 mL 6.0% NaOCl Supernatants	30 min, 120 min, 24 h	1:1; 1:2; 1:4; 1:8; 1:16, and 1:32
3T3 or HPLFs	1 mL 2.0% CHX + 1 mL 6.0% NaOCl Precipitates	30 min, 120 min, 24 h	1:1000, 1:2000, 1:4000, 1:8000, and 1:16,000

CHX, chlorhexidine; HPLFs, human periodontal ligament fibroblasts; NaOCl, sodium hypochlorite.

chromatography (HPLC), proton nuclear magnetic resonance spectroscopy, thin-layer chromatography, infrared spectroscopy, and gas chromatography–mass spectrometry (22).

Studies showed that PCA can be rapidly oxidized to p-chloronitrobenzene (PCN) (15, 23, 24), and both compounds have been proven carcinogenic in animals (25, 26). The present study aimed to investigate the stability of PCA in the presence of the oxidant effects of NaOCl and to evaluate the toxic effects of the CHX and NaOCl reaction mixture and degradative by-products on 2 cell types (3T3-Swiss Albino mouse fibroblasts cells and human periodontal ligament fibroblasts [HPLFs]).

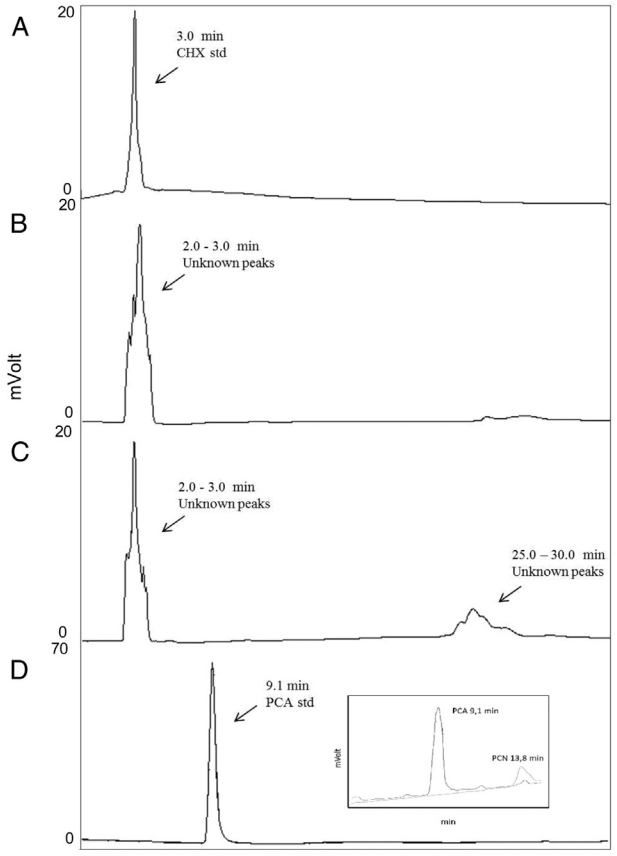

Figure 1. (*A*) The chromatographic profile of CHX. (*B*) The chromatographic profile of CHX in the presence of 0.12% NaOCl. (*C*) The chromatographic profile of CHX in the presence of 0.48% NaOCl (6%). (*D*) The chromatographic profile of PCA. (*Insert*) The chromatographic profile of PCN.

Materials and Methods

Unless otherwise specified, all chemicals and reagents used in this study (cell culture grade) were obtained from Sigma-Aldrich, St Louis, MO.

Preparation of CHX/NaOCl and PCA/NaOCl Reaction Mixtures

Reaction mixtures were prepared by the addition of 1 mL 2% CHX to 4 different volumes of 6.0% NaOCl as follows: group 1, 20 μL NaOCl (the final NaOCl concentration was 0.12%); group 2: 40 μL NaOCl (the final NaOCl concentration was 0.24%); group 3: 80 μL NaOCl (the final NaOCl concentration was 0.48%); and group 4: 160 μL NaOCl (the final NaOCl concentration was 0.96%).

PCA was dissolved in methanol at a concentration of 5 mg/mL, and 1 mL of this solution was mixed with 20 μL of 6% NaOCl (group 1), 40 μL (group 2), 80 μL (group 3), and 160 μL (group 4). A reaction mixture between 0.2 mL 0.2% NaOH (pH = 9.0) and PCA (5 mg/mL in CH_3OH) was also prepared to verify the effect of high alkalinity in the reaction product formation.

A standard solution with PCN was analyzed to identify the chromatographic peak. The specimens were centrifuged (13,400*g* for 5 minutes), and the supernatants were collected and filtered using an ion chromatography Acrodisc (13-mm syringe filter [0.2-μm Supor PES membrane; Pall Italia Srl, Buccinasco, Milan, Italy]). Precipitates were resuspended in 1 mL CH_3OH and filtered using the system described previously. Before high-performance liquid chromatographic analysis, both supernatants (SNs) and precipitates (PRs) were further diluted in CH_3OH (from 4-fold to 20-fold).

HPLC. High-performance liquid chromatographic separations were performed using a Discovery HS C18 column (250 mm × 4.6 mm, 5 μm [SUPELCO; Supelco Park, Bellefonte, PA]) at a flow rate of 0.7 mL/min with detection at 214 nm. The mobile phase was constituted by a mixture of water (A) and acetonitrile (CH_3CN) (B) using a gradient elution starting from 50% (B) to 70% (B) for 10 minutes and then to 85% (B) for 5 minutes. CHX, PCA, and PCN chromatographic conditions were obtained using solutions prepared with standards before the analysis of reaction mixtures. Each experiment was repeated 3 times.

Cytotoxicity Evaluation on HPLFs and 3T3 Fibroblasts.

Cell culture. HPLFs were obtained and cultured as discussed in a previous study (27). Mouse 3T3 fibroblasts (Swiss albino mouse cell line; Istituto Zooprofilattico, Brescia, Italy) were cultured in Dulbecco modified Eagle medium supplemented with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (10 mmol/L), glucose (1 g/L), $NaHCO_3$ (3.7 g/L), penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% fetal calf serum. Both cell lines were

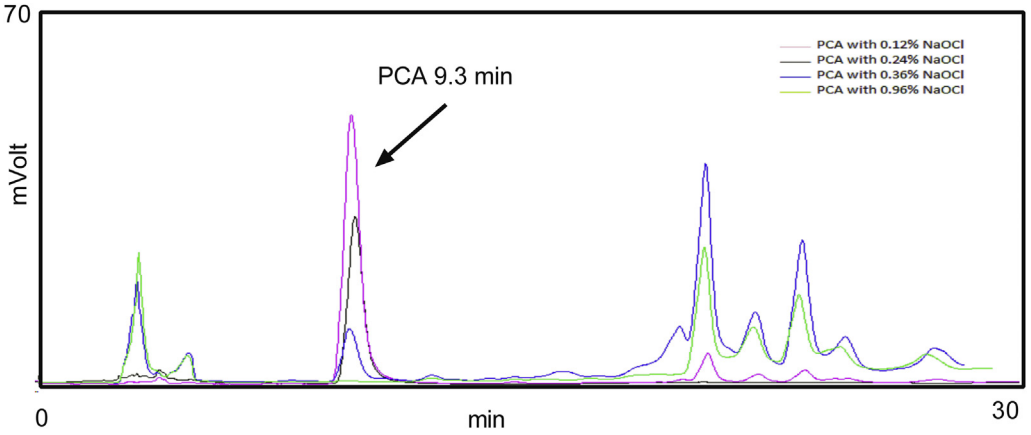

Figure 2. Chromatographic profiles of PCA with 0.12% NaOCl (*pink*), PCA with 0.24% NaOCl (*black*), PCA with 0.48% NaOCl (*blue*), and PCA with 0.96% NaOCl (*green*).

cultured in tissue culture flasks and incubated in 5% CO_2 atmosphere at 37°C. The media were changed every third day.

Cell treatment. HPLFs and 3T3 fibroblasts (1×10^4) in 0.2 mL Dulbecco modified Eagle medium were seeded into each well of a 96-well tissue culture plate (Costar, Cambridge, MA), cultured to a subconfluent monolayer, and incubated in 5% CO_2 at 37°C for 24 hours.

The reaction mixtures were prepared by mixing 1.0 mL 2% CHX with 1 mL 6% NaOCl. The mixtures were centrifuged (at 13,400*g* for 5 minutes), and the PRs were resuspended in 0.2 mL dimethyl sulfoxide; then, both fibroblast cell lines were incubated with PR and SN dilutions as mentioned in Table 1 for 30 minutes, 2 hours, and 24 hours. Dimethyl sulfoxide concentration was kept at a percentage of <0.1% throughout the experiment. After incubations (at 37°C in a humidified atmosphere), the cytotoxicity induced by PRs and SNs was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT).

Cytotoxicity evaluation. The MTT test was performed according to Wataha et al (28). At each time interval, the MTT solution (20 μL) in phosphate-buffered saline (5 mg/mL) was added to the medium (0.2 mL), and after incubation (4 hours at 37°C), the intracellular formazan crystals produced were solubilized with a solution of HCl in isopropanol (4×10^{-2} N, 0.2 mL). The optical density (OD) of the solution contained in each well was determined using an automatic microplate photometer (Packard Spectracount; Packard BioScience Company, Meriden, CT) at a wavelength of 570 nm.

Each experiment was performed in sextuplicate and repeated 4 times, and the cell mortality was calculated according to the following equation (27):

$$\text{Cell mortality} = \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \times 100$$

Specimens were rated as slightly, moderately, or severely cytotoxic when the toxic effects, relative to controls, were less than 30%, between 30% and 60%, or greater than 60%, respectively (29).

Statistical Analysis

Data were expressed as mean ± standard deviation, taking into consideration at least 3 different experiments performed in duplicates. The means were compared by analysis of variance followed by a multiple comparison (if the difference is significant) of means using the Student-Newman-Keuls test. The level of significance was set at 0.05.

Results

High-performance Liquid Chromatographic Analysis of Supernatants of CHX/NaOCl and PCA/NaOCl Reaction Mixtures

High-performance liquid chromatographic analysis of the CHX standard (2% in H_2O, pH = 8.00) showed the presence of a chromatographic signal with a 3.0-minute retention time (RT) (Fig. 1*A*). Other chromatographic signals were observed before and after the peak of CHX when 20 μL NaOCl (6%) was mixed with CHX (Fig. 1*B*). At a volume of 80 μL NaOCl, the chromatographic peaks were decreased in number, and other signals were evident with the RT ranging between 20 and 25 minutes (Fig. 1*C*). Under these experimental conditions, the formation of chromatographic peaks at the RT of PCA was not observed (Fig. 1*C* and *D*), but a signal ascribed to PCN was elicited (Fig. 1*D* insert) with other degradation products.

The high-performance liquid chromatographic signal intensity of PCA was decreased when NaOCl was added and disappeared completely after the addition of 160 μL NaOCl (0.96% final concentration of NaOCl) (Figs. 2 and 3). At the same time, signals with an RT between 20 and 25 minutes were produced (Fig. 2), which is similar to what

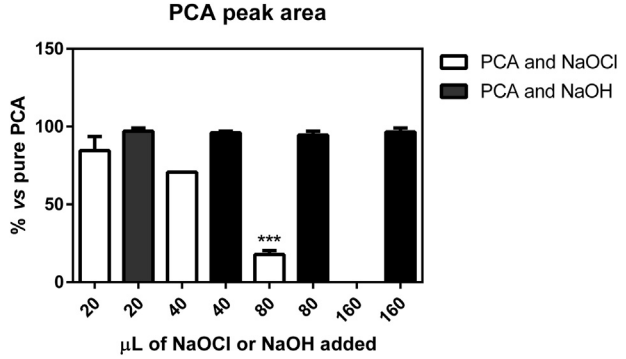

Figure 3. PCA peak area in the presence of different concentrations of NaOCl (or NaOH) versus pure PCA. ***$P < 0.001$

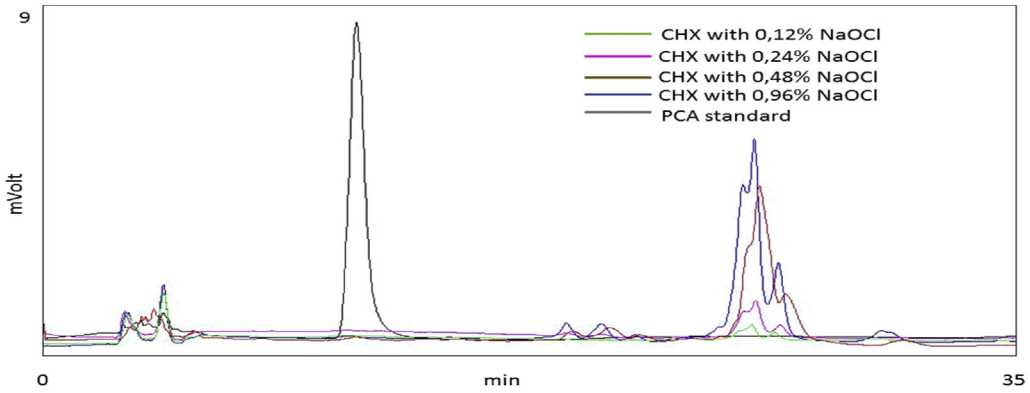

Figure 4. Chromatographic profiles of CHX with 0.12% NaOCl (*green*), CHX with 0.24% NaOCl (*black*), CHX with 0.48% NaOCl (*pink*), and CHX with 0.96% NaOCl (*blue*).

was observed for CHX (Fig. 1*C*). The addition of NaOH to PCA did not provoke PCA degradation (Fig. 3).

High-performance Liquid Chromatographic Analysis of Precipitates of CHX/NaOCl and PCA/NaOCl Reaction Mixtures

High-performance liquid chromatographic analysis of PRs of CHX/NaOCl reaction mixtures showed the presence of many chromatographic signals with 2–3 minute and 20–25 minute RTs (Fig. 4). The intensities of all peaks increased when the NaOCl concentration increased. High-performance liquid chromatographic analysis of the PRs of PCA/NaOCl reaction mixtures showed signals with 2–3 minute and 20–25 minute RTs (Fig. 5), as observed in the CHX/NaOCl reaction mixtures.

Cytotoxic Effects of the Brown Precipitates Formed by CHX/NaOCl Reaction Mixtures

Results showed that the cytotoxic effects of the dissolved brown precipitate were slight on both cell lines (HPLFs and 3T3 fibroblasts). Figure 6*A* and *B* show the cell mortality values at all time intervals.

Cytotoxic Effects of Supernatants from CHX/NaOCl Reaction Mixtures

The results showed severe cytotoxic effects on HPLFs after 30 minutes of incubation (Fig. 7*A* and *B* and Table 2). On the other hand, the cytotoxic effects of these supernatants on the mouse 3T3 fibroblasts were obviously dependent on the reagent concentrations. Furthermore, the toxic effects induced by other dilutions increased over time (Fig. 7).

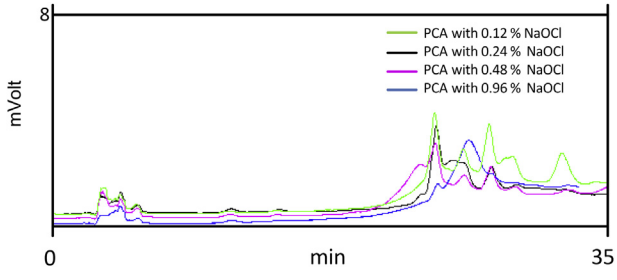

Figure 5. Chromatographic profiles of PCA with 0.12% NaOCl (*green*), PCA with 0.24% NaOCl (*black*), PCA with 0.48% NaOCl (*pink*), and PCA with 0.96% NaOCl (*blue*).

Discussion

The literature shows controversies with regard to the formation of toxic PCA when NaOCl is mixed with CHX (19, 22, 30). Some studies observed the presence of PCA in brown precipitates (19, 20, 30), whereas others did not (21, 22). These contrasting results may be attributed to the different experimental conditions, potential artifacts of the analytic method, and/or the instability of PCA in the presence of NaOCl shown in the present study.

CHX reacts with NaOCl forming small molecules (19), some of which are insoluble in water and thus precipitate. In the present study, HPLC was used to analyze both SNs and PRs from the reaction mixtures. Several chromatographic signals derived from CHX degradation were observed, but no PCA was detected. This might be attributed to the oxidant characteristics of NaOCl that rapidly transform PCA to PCN and other reaction-derived by-products (Figs. 2 and 5). For this reason, PCA may become undetectable as shown in previous studies.

To verify this hypothesis, we evaluated the stability of PCA in the presence of increased concentrations of NaOCl, in both SNs and PRs, and the results indicated that PCA likely undergoes an oxidation to PCN traces, which were observed in CHX/NaOCl and PCA/NaOCl reaction mixtures (Fig. 1*D*). Notably, the degradation is not pH dependent as observed with the addition of NaOH.

The chromatograms obtained from the CHX/NaOCl and PCA/NaOCl reaction mixtures showed some signals having the same RT in both conditions (about 20–25 minutes). This result confirms that PCA has a short half-life because of the presence of hypochlorite giving rise to derivative by-products.

The biological impact of an endodontic irrigating solution is significant because toxicity against the periapical tissue can delay wound healing and worsen tissue damages; thus, cytotoxicity assays were performed. Results of the present study are consistent with 1 investigation that showed that the precipitate is more cytotoxic than NaOCl and CHX alone when implanted under the skin of an animal model (31). This is contradicted with a recent study that showed that the NaOCl/CHX combination exerted less cytotoxic effects on human lung fibroblasts than the single irrigants (32). Apart from different methodological procedures, it does not seem appropriate to correlate such findings with the clinical situation because cell types that would be in contact with this combination in clinical

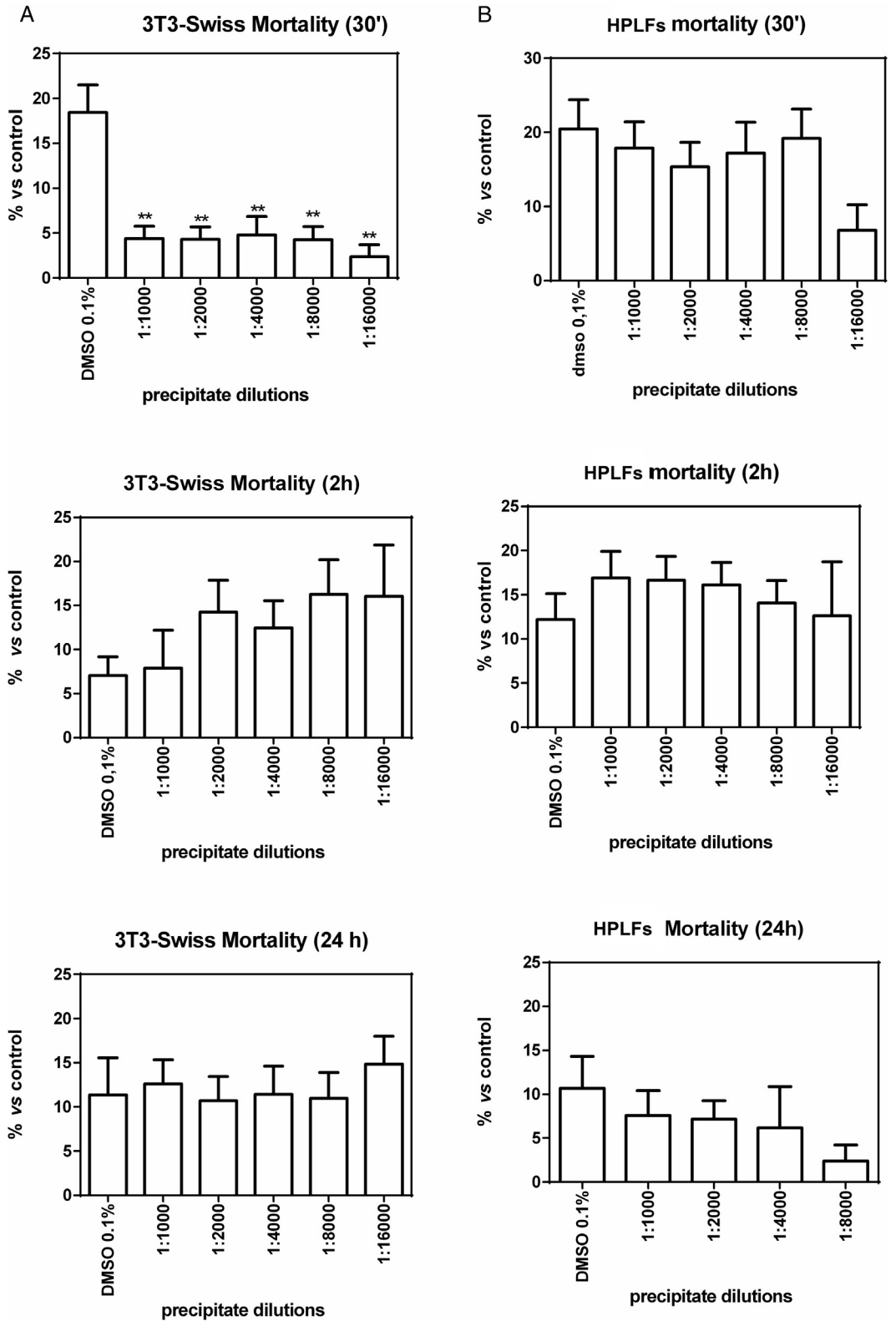

Figure 6. Cytotoxic effects induced by PRs formed by reaction mixtures between CHX and NaOCl on (*A*) 3T3 Swiss and (*B*) HPLFs. $^{**}P < 0.01$

settings may react differently, which has been shown in the present study with supernatants from CHX/NaOCl reaction mixtures. This is why a cell type that is closely related to the human tooth (HPLFs) was 1 of the 2 cell lines chosen for the present study.

Experiments on CHX/NaOCl reaction mixtures at different dilutions in both SNs and PRs (which had not yet been reported in the scientific literature) showed that the mortality induced by PRs was lower (10%–20%) than that induced by SNs in all

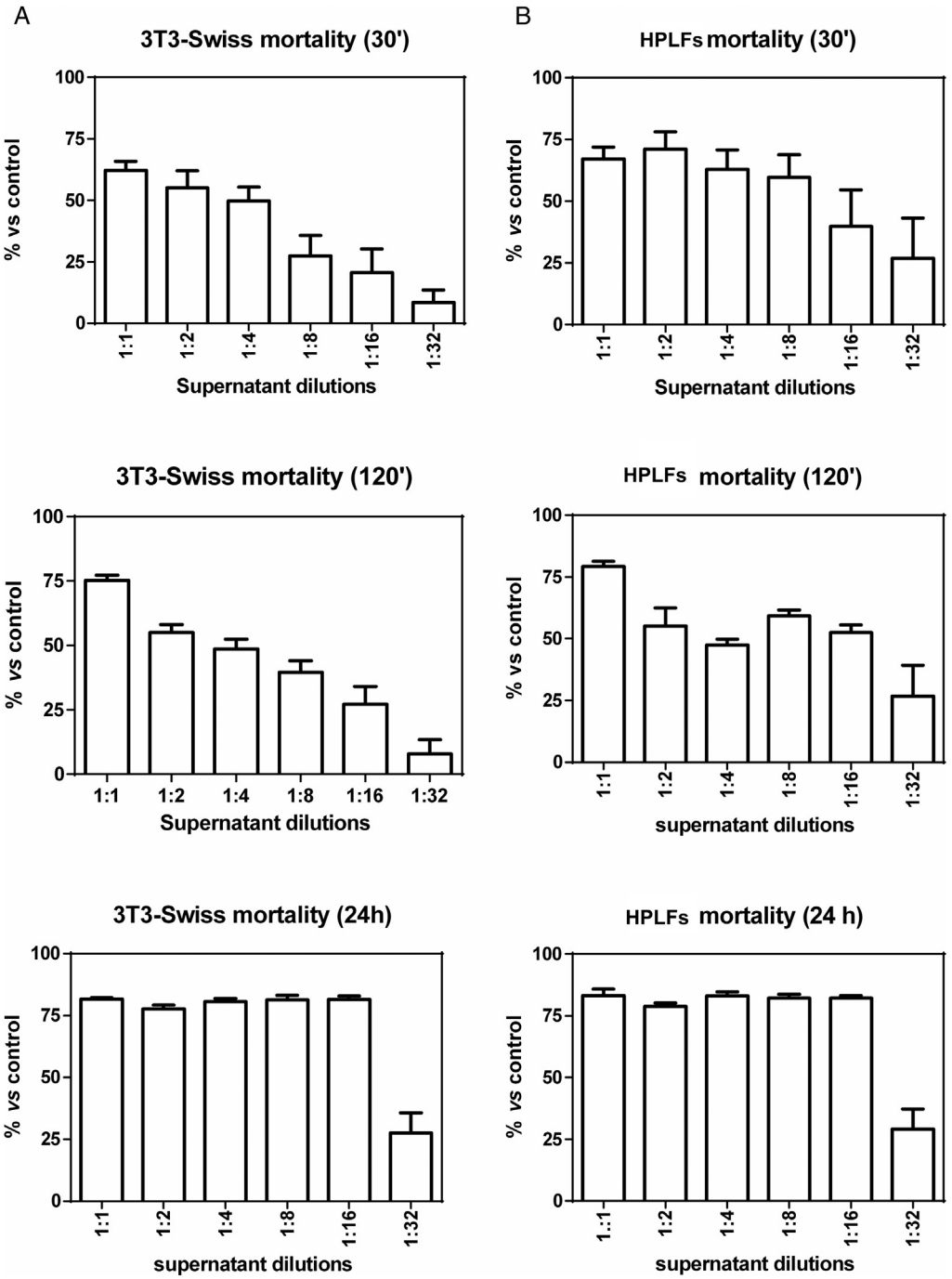

Figure 7. Cytotoxic effects induced by SNs from the reaction mixtures between CHX and NaOCl on (*A*) 3T3 Swiss and (*B*) HPLFs.

experimental conditions. Conversely, when SNs were considered, the mortality was dependent on the concentration and incubation time, especially with mouse 3T3 fibroblasts (Fig. 7). This finding indicates the CHX/NaOCl reaction mixture is composed of a variety of chemical components that have wide ranges of potential cytotoxic effects. This also may explain inconsistencies among different cytotoxicity studies (31, 32).

Based on the previous discussion, incorporating thorough intermediate flushes between each irrigant followed by drying of the canal before the next solution is essential to prevent the formation of by-products (17, 18). A number of intermediate canal flushes, such as distilled water, saline, alcohol, and others, have been recommended to remove residues of NaOCl before the use of CHX (17, 18, 30, 33). However, a recent study showed that intermediate flushes failed to prevent the precipitation of residues on canal walls (33), probably because of the complex canal anatomy, which may complicate the complete removal of NaOCl residues. Therefore, practitioners should follow preventive guidelines to minimize precipitate formation, and NaOCl/CHX combination should be used only when indicated (18).

TABLE 2. Statistical Analysis of Cytotoxic Effects Induced by Chlorhexidine/Sodium Hypochlorite Reaction Mixtures on 3T3 Swiss (A) and Human Periodontal Ligament Fibroblasts (B) Expressed as Dilution Ratios

Incubation time	1:32 vs 1:1	1:32 vs 1:2	1:32 vs 1:4	1:32 vs 1:8	1:32 vs 1:16	1:16 vs 1:1	1:16 vs 1:2	1:16 vs 1:4	1:16 vs 1:8	1:8 vs 1:1	1:8 vs 1:2	1:8 vs 1:4	1:4 vs 1:1	1:4 vs 1:2	1:2 vs 1:1
A															
30 min	‡	‡	‡	NS	NS	†	†	*	NS	†	*	*	NS	NS	Ns
2 h	‡	‡	‡	‡	*	‡	†	*	NS	‡	†	NS	‡	NS	‡
24 h	‡	‡	‡	‡	‡	NS	NS	NS	NS	NS	NS	NS	NS	NS	Ns
B															
30 min	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
2 h	‡	*	*	*	*	*	NS	NS	NS	*	NS	NS	NS	NS	*
24 h	‡	‡	‡	‡	‡	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

NS, not significant.
*$P < .05$.
†$P < .01$.
‡$P < .001$.

Conclusions

The discrepancy of the data in the literature could be caused by the instability of the PCA in the presence of NaOCl. The favorable cytotoxic profile of the CHX/NaOCl reaction mixture could be ascribed to the oxidant effects of NaOCl on reaction products.

Acknowledgments

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

References

1. Siqueira JF Jr. Aetiology of root canal treatment failure: why well-treated teeth can fail. Int Endod J 2001;34:1–10.
2. Peters OA, Schönenberger K, Laib A. Effects of four Ni-Ti preparation techniques on root canal geometry assessed by micro computed tomography. Int Endod J 2001;34:221–30.
3. Zehnder M. Root canal irrigants. J Endod 2006;32:389–98.
4. Hand RE, Smith ML, Harrisen JW. Analysis of the effect of dilution of the necrotic tissue dissolution property of sodium hypochlorite. J Endod 1978;4:60–4.
5. Rosenfeld EF, Jemes GA, Buckner SB. Vital pulp tissue response to sodium hypochlorite. J Endod 1978;4:140–6.
6. Byström A, Sundquist G. Bacteriological evaluation of the effect of 0.5% sodium hypochlorite in endodontic therapy. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1983;55:307–12.
7. Yesilsey C, Whitaken E, Cleveland D, et al. Antimicrobial and toxic effects of established and potential root canal irrigants. J Endod 1995;21:513–5.
8. Barnard D, Davies J, Figdor D. Susceptibility of Actinomyces israelii to antibiotics, sodium hypochlorite and calcium hydroxide. Int Endod J 1996;29:320–6.
9. Ayhan H, Sultan N, Cirak M. Antimicrobial effects of various endodontic irrigants on selected microorganism. Int Endod J 1999;32:99–102.
10. Marco S, Rullo R, Albino A, et al. The thioredoxin system in the dental caries pathogen Streptococcus mutans and the food-industry bacterium Streptococcus thermophilus. Biochimie 2013;95:2145–56.
11. Havlíková L, Matysová L, Nováková L, et al. HPLC determination of chlorhexidine gluconate and p-chloroaniline in topical ointment. J Pharm Biomed Anal 2007;43:1169–73.
12. Emilson CG. Susceptibility of various microorganisms to chlorhexidine. Scand J Dent Res 1977;85:255–65.
13. Junco MP, Baca P. Métodos de control de placa bacteriana. In: Cuenca Sala E, Baca García P, eds. *Odontología Preventiva y Comunitaria. Principios, Métodos y Aplicaciones*. Barcelona: Masson; 2005:87–104.
14. Vivacqua-Gomes N, Ferraz CC, Gomes BP, et al. Influence of irrigants on the coronal microleakage of laterally condensed gutta-percha root fillings. Int Endod J 2002;35:791–5.
15. Bui TB, Baumgartner JC, Mitchell JC. Evaluation of the Interaction between sodium hypochlorite and chlorhexidine gluconate and its effect on root dentin. J Endod 2008;34:181–5.
16. Gupta H, Kandaswamy D, Manchanda SK, Shourie S. Evaluation of the sealing ability of two sealers after using chlorhexidine as a final irrigant: an *in vitro* study. J Conserv Dent 2013;16:75–8.
17. Vouzara T, Koulaouzidou E, Ziouti F, Economides N. Combined and independent cytotoxicity of sodium hypochlorite, ethylenediaminetetraacetic acid and chlorexhidine. Int Endod J 2016;49:764–73.
18. Ahmed HMA, Abbott PV. Discolouration potential of endodontic procedures and materials: a review. Int Endod J 2012;45:883–97.
19. Basrani BR, Manek S, Sodhi RN. Interaction between sodium hypochlorite and chlorhexidine gluconate. J Endod 2007;33:966–9.
20. Basrani BR, Manek S, Mathers D, et al. Determination of 4-chloroaniline and its derivatives formed in the interaction of sodium hypochlorite and chlorhexidine by using gas chromatography. J Endod 2010;36:312–4.
21. Thomas JE, Sem DS. An *in vitro* spectroscopic analysis to determine whether parachloroaniline is produced from mixing sodium hypochlorite and chlorhexidine. J Endod 2010;36:315–7.
22. Orhan EO, Irmak O, Hur D, et al. Does Para-chloroaniline really form after mixing sodium hypochlorite and chlorhexidine? J Endod 2016;42:455–9.
23. Below H, Lehan N, Kramer A. HPLC determination of the antiseptic agent chlorhexidine and its degradation products 4-chloroaniline and 1-chloro-4-nitrobenzene in serum and urine. Mikrochim Acta 2004;146:129–35.

24. Zong Z, Kirsch LE. Studies on the instability of chlorhexidine, part I: kinetics and mechanisms. J Pharm Sci 2012;101:2417–27.
25. Chhabra RS, Huff JE, Hasema JK, et al. Carcinogenicity of p-chloroaniline in rats and mice. Food Chem Toxicol 1991;29:119–24.
26. Matsumoto M, Aiso S, Senoh H, et al. Carcinogenicity and chronic toxicity of para-chloronitrobenzene in rats and mice by two-year feeding. J Environ Pathol Toxicol Oncol 2006;25:571–84.
27. Gambarini G, Plotino G, Grande NM, et al. *In vitro* evaluation of the cytotoxicity of FotoSan light-activated disinfection on human fibroblasts. Med Sci Monit 2011;17: MT21–5.
28. Wataha JC, Hanks CT, Craig RG. *In vitro* synergistic, antagonistic, and duration of exposure effects of metal cations on eukaryotic cells. J Biomed Mater Res 1992; 26:1297–309.
29. Sletten GB, Dahl JE. Cytotoxic effects of extracts of compomers. Acta Odontol Scand 1999;57:316–22.
30. Krishnamurthy S, Sudhakaran S. Evaluation and prevention of the precipitate formed on interaction between sodium hypochlorite and chlorhexidine. J Endod 2010;36: 1154–7.
31. Cintra LT, Watanabe S, Samuel RO, et al. The use of NaOCl in combination with CHX produces cytotoxic product. Clin Oral Investig 2014;18:935–40.
32. Prado M, Santos Júnior HM, Rezende CM, et al. Interactions between irrigants commonly used in endodontic practice: a chemical analysis. J Endod 2013;39:505–10.
33. Magro MG, Kuga MC, Aranda-Garcia AJ, et al. Effectiveness of several solutions to prevent the formation of precipitate due to the interaction between sodium hypochlorite and chlorhexidine and its effect on bond strength of an epoxy-based sealer. Int Endod J 2015;48:478–83.