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

*Giuseppina Nocca, PhD,*[*†] *Hany Mohamed Aly Ahmed, BDS, HDD (Endo), PhD,*[‡] *Giuseppe Ettore Martorana, MD,*[*] *Cinzia Callà, MD,*[*] *Gianluca Gambarini, DDS,*[§] *Sandro Rengo, DDS,*[¶] *and Gianrico Spagnuolo, DDS, PhD*[¶]

## 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;■:1–8)*

## 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

From the *Institute of Biochemistry and Clinical Biochemistry, Università Cattolica del Sacro Cuore, Largo Francesco Vito, Rome, Italy; †Istituto di Chimica del Riconoscimento Molecolare, Consiglio Nazionale delle Ricerche, Largo Francesco Vito, Rome, Italy; ‡Department of Restorative Dentistry, Faculty of Dentistry, University of Malaya, Kuala Lumpur, Malaysia; §Department of Oral and Maxillo Facial Sciences, Sapienza, Università di Roma, Rome, Italy; and ¶Department of Neurosciences, Reproductive and Odontostomatological Sciences, Università di Napoli Federico II, Naples, Italy.

Address requests for reprints to Dr Gianrico Spagnuolo, Department of Neurosciences, Reproductive and Odontostomatological Sciences, Università di Napoli Federico II, Via S. Pansini, 5-80131 Naples, Italy. E-mail address: gianrico.spagnuolo@gmail.com

0099-2399/$ - see front matter



http://dx.doi.org/10.1016/j.joen.2017.04.025

**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]).

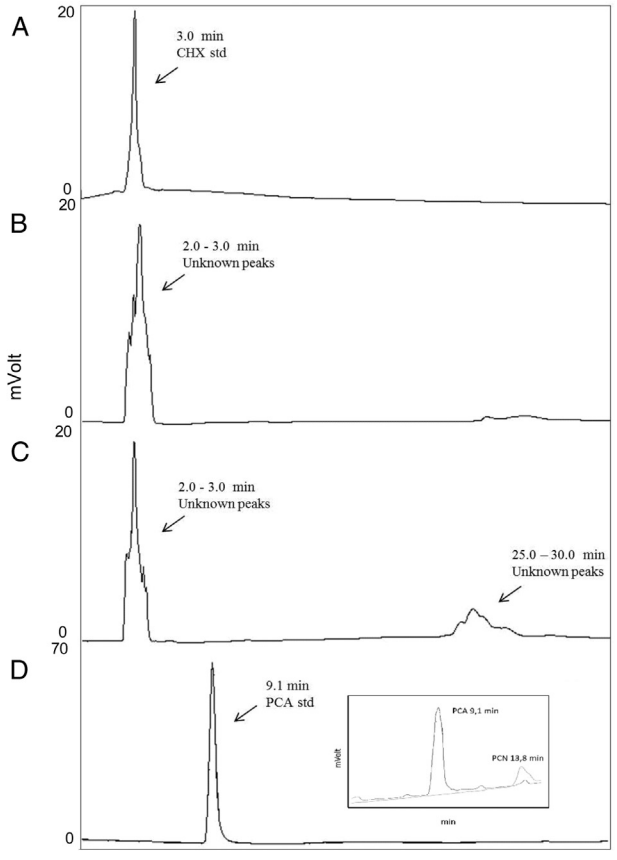


**Figure 1.** (*A*) The chromatographic profile of CHX. (*B*) The chromatographic profile of CHX in the presence of 0.12% NaOCl. (*C*) The chromatographic profile of CHX in the presence of 0.48% NaOCl (6%). (*D*) The chromatographic profile of PCA. (*Insert*) The chromatographic profile of PCN.

## 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 $\mu$L NaOCl (the final NaOCl concentration was 0.12%); group 2: 40 $\mu$L NaOCl (the final NaOCl concentration was 0.24%); group 3: 80 $\mu$L NaOCl (the final NaOCl concentration was 0.48%); and group 4: 160 $\mu$L 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 $\mu$L of 6% NaOCl (group 1), 40 $\mu$L (group 2), 80 $\mu$L (group 3), and 160 $\mu$L (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-$\mu$m 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 $\mu$m [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 $\mu$g/mL), and 10% fetal calf serum. Both cell lines were