

An *In Vitro* Spectroscopic Analysis to Determine the Chemical Composition of the Precipitate Formed by Mixing Sodium Hypochlorite and Chlorhexidine

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Abstract

Introduction: The purpose of this *in vitro* study was to determine the chemical composition of the precipitate formed by mixing sodium hypochlorite (NaOCl) and chlorhexidine (CHX) and the relative molecular weight of the components. **Methods:** Using commercially available CHX gluconate, a 2% solution was formed and mixed in a 1:1 ratio with commercially available NaOCl producing a brown precipitate. The precipitate as well as a mixture of precipitate and pure CHX diacetate was then analyzed using one-dimensional and two-dimensional NMR spectroscopy. **Results:** The one-dimensional and two-dimensional NMR spectra were fully assigned in terms of chemical shifts of all proton and carbon atoms in intact CHX. This permitted identification of two major CHX breakdown products, neither of which are parachloroaniline (PCA). Both products are related to PCA in that they are parasubstituted benzene compounds. Based on NMR data and a proposed mechanism of CHX breakdown, the products appear to be parachlorophenylurea (PCU) and parachlorophenylguanidyl-1,6-diguanidyl-hexane (PCGH). **Conclusions:** Based on this *in vitro* study, the precipitate formed by NaOCl and CHX is composed of at least two separate molecules, all of which are smaller in size than CHX. Along with native CHX, the precipitate contains two chemical fragments derived from CHX (PCU and PCGH), neither of which are PCA. (*J Endod* 2011;37:983–988)

Key Words

Chlorhexidine, nuclear magnetic resonance, parachloroaniline, sodium hypochlorite

Bacteria in the root canal system provoke the formation of periapical inflammatory lesions (1). The goal of root canal therapy is to remove the bacteria along with the inflamed or necrotic pulp. Although biomechanical cleaning and shaping of the root canal greatly reduces the number of bacteria (2), bacteria cannot be completely removed (3). Chemical debridement in the form of various irrigants is performed to aid in the removal of residual debris, necrotic tissue, and bacteria.

Because of its broad-spectrum antimicrobial action and tissue-dissolving properties, sodium hypochlorite (NaOCl) at various concentrations is the most common endodontic irrigant used (4–6). Despite its germicidal abilities, NaOCl in high concentration is cytotoxic and can cause necrosis of periapical tissues (5, 7, 8). NaOCl is not a substantive microbial agent (9). These troubles have led clinicians and researchers to explore alternative irrigants.

Chlorhexidine gluconate (CHXg) is a broad-spectrum antimicrobial agent that has been advocated as an effective medication in endodontic treatment (10, 11). When used as an endodontic irrigant, chlorhexidine (CHX) has an antimicrobial efficacy comparable to that of NaOCl but lacks the cytotoxic effects (8, 12). CHX has also been shown to have antimicrobial substantivity in root dentin (13–15).

A drawback of CHX is that it lacks the ability to dissolve organic matter. For this reason, CHX is often used in conjunction with NaOCl. Kuruville and Kamath (5) suggested that the antimicrobial effect of NaOCl and CHX in combination was better than that of either component alone. When mixed, however, NaOCl and CHX produce a brown precipitate that stains the walls of the pulp chamber and has been reported to be difficult to remove (16). It has also been reported by previous researchers that this precipitate contains the cytotoxin parachloroaniline (PCA) (17–19). However, a recent study by Thomas and Sem (20) showed that PCA was not produced in any measurable quantity.

Nuclear magnetic resonance (NMR) spectroscopy is one of the principal techniques used to structurally characterize molecules based on chemical shift values and couplings between atoms as well as to determine purity of mixtures of molecules based on relative signal intensities. By exciting energy level transitions that are very low in energy, even the most fragile bonds remain intact. The presence or absence of specific molecules in a mixture can then be determined by comparing the mixture's NMR spectrum with spectra of pure compounds. For more complicated molecules, two-dimensional (2D) NMR spectroscopy such as correlation spectroscopy (COSY) provides valuable structural information by evaluating three bond couplings (eg, from a proton through its carbon, to the adjacent carbon, then to that carbon's proton). When dealing with mixtures of compounds, another 2D NMR spectroscopy technique known as diffusion-ordered spectroscopy (DOSY) can allow the different compounds to be separated based on their differing diffusion coefficients, which are a function of molecular size. Heteronuclear multiple quantum coherence (HMQC) experiments can also be used as an adjunct to allow for the assignment of carbon chemical shifts by correlating ^1H and ^{13}C chemical shifts. Such additional experiments permit one to go beyond qualitatively comparing spectra (eg, between PCA and the precipitate) to actually identify what chemical is produced, which was the focus of this study.

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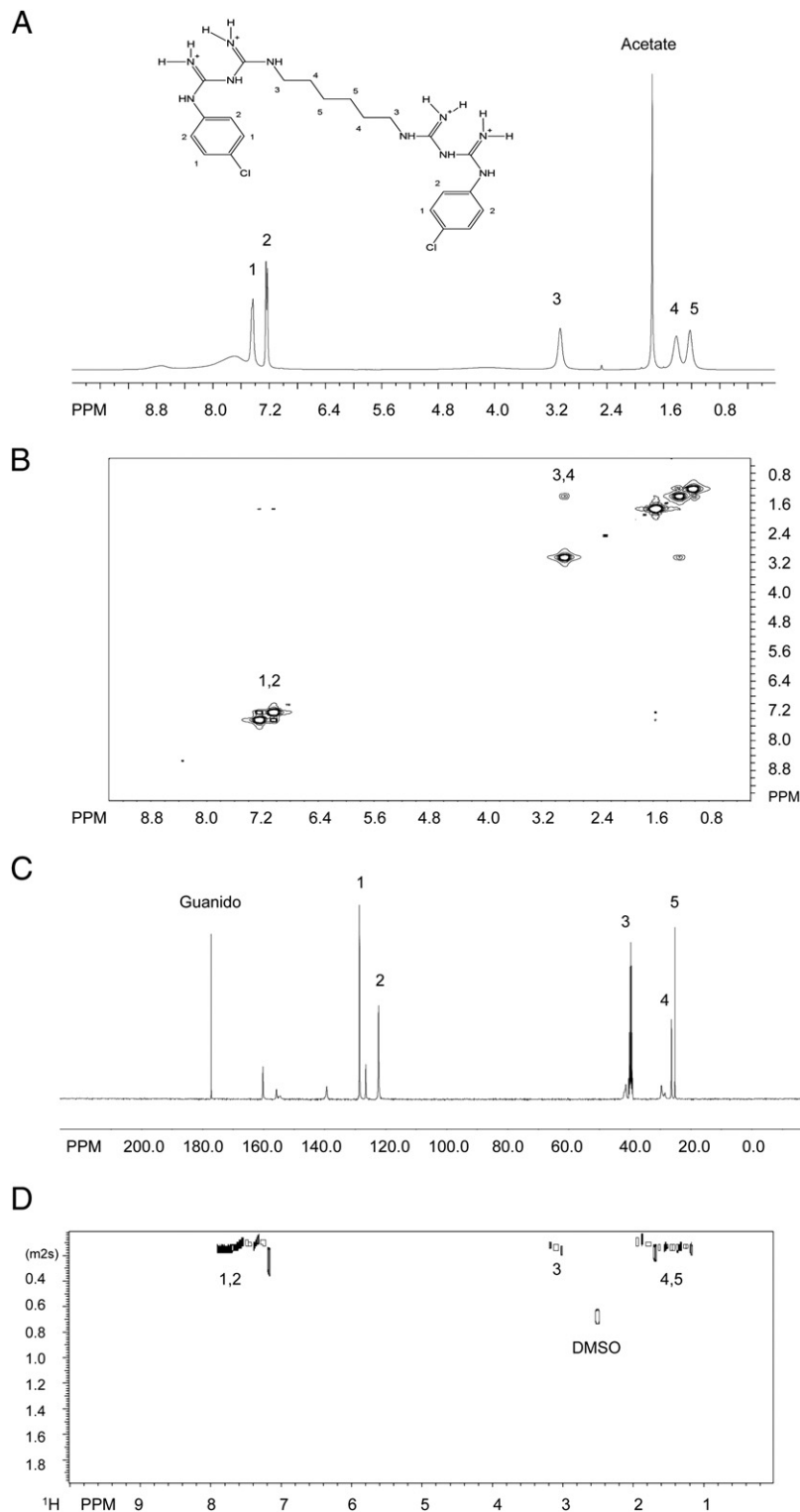


Figure 1. NMR spectra for pure CHX diacetate. (A) ^1H NMR spectrum of CHX, with protons assigned according to the numbering scheme shown on the chemical structure of CHX (in the insert). (B) 2D ^1H - ^1H COSY spectrum showing cross peaks that were used to make the assignments for proton pairs (1/2) and (3/4) according to the numbering scheme used in panel A. (C) 1D ^{13}C NMR spectrum of CHX with carbon assignments based on ^1H - ^{13}C HMQC spectra. (D) 2D DOSY spectrum showing that only one species is present and diffusing with a diffusion coefficient of $<0.5 \times 10^{-10} \text{ m}^2/\text{s}$.

Although there has been much speculation as to whether PCA is or is not present in the precipitate formed by CHX and NaOCl, to date the composition of the precipitate has not been completely characterized. The purpose of this *in vitro* study was to determine the chemical composition of the precipitate formed by NaOCl and CHX.

Materials and Methods

A commercially available sample of CHXg (Sigma-Aldrich, St Louis, MO) was analyzed with ^1H NMR spectroscopy (400-MHz Varian NMR System acquiring 32 scans/spectrum) with perdeuterated dimethyl sulfoxide (d_6 -DMSO) as a solvent.

A 2% aqueous solution of CHXg was prepared by mixing 2.5 mL 20% CHXg with 22.5 mL deionized H_2O . This solution was warmed to 37°C and mixed with 25 mL of 5.25% NaOCl and stirred continuously. The brown precipitate formed immediately. Two 1.5-mL samples were taken and placed in 1.5-mL microfuge tubes and centrifuged at 14,000 rpm for 2.5 minutes. The precipitate solid was removed and dissolved in 1.0 mL of d_6 -DMSO; 100 μL of 0.5 g/mL pure CHX diacetate (CHXa) dissolved in d_6 -DMSO was added to one sample. One-dimensional (^1H , ^{13}C) and 2D (COSY, DOSY, and HMQC) NMR spectra were then collected for each of the samples at 25°C . Resulting spectra were fully assigned in terms of chemical shifts of all proton and carbon atoms in intact CHX, which permitted the identification of CHX breakdown products.

Results

Before the analysis of CHX breakdown products can be undertaken, full NMR characterization of pure CHX (as the acetate salt) was performed as summarized in Figure 1. A prominent COSY cross peak in Figure 1B establishes the connection between the deshielded N-attached CH_2 linker protons (3, at 3.08 ppm) and the adjacent CH_2 protons in position 4 (1.43 ppm). This leaves the $-\text{CH}_2$ protons at position 5 as assigned to the most up-field resonance at 1.24 ppm. These proton chemical shift assignments allow assignment of all chemical shifts in the 1D ^{13}C spectrum (Fig. 1C) based on the 2D ^1H - ^{13}C HMQC spectrum (not shown). The DOSY spectrum in Figure 1D shows all expected peaks for protons 1 through 5 for a molecule diffusing with a diffusion coefficient of $<0.5 \times 10^{-10} \text{ m}^2/\text{s}$.

CHX was then treated with NaOCl to form a precipitate, and that precipitate (dissolved in DMSO) was analyzed using DOSY spectra. There are two products (ignoring solvent and gluconate signals), both of which are lower in molecular weight than the original CHX because they have larger diffusion coefficients of around 1 to $2.5 \times 10^{-10} \text{ m}^2/\text{sec}$ (Fig. 2B). These breakdown products have aromatic signals around 7 to 8 ppm, and one also has protons with chemical shifts of 4 ppm and 2.5 ppm. These chemical shifts are consistent with at least one of the breakdown products still possessing the aliphatic linker (ie, chlorophenylguanidyl-1,6-diguanidyl-hexane [PCGH]). The other breakdown product seems to be lacking the aliphatic protons, which is consistent with the linker being absent (ie, parachlorophenylurea [PCU]). The DOSY spectrum also shows the three characteristic NMR resonances for undegraded CHX at the top of Figure 2B. To confirm that these signals are in fact from undegraded CHX, pure CHX was spiked into the sample. The resulting DOSY spectrum (Fig. 2D) shows an increase in intensity for these signals, consistent with them having been from residual undegraded CHX.

In a repeat of the CHX + NaOCl experiment (using CHXg), the COSY spectrum of the precipitate (Fig. 3A) shows two amides from the guanido with connectivity to the linker CH_2 - (and a third minor species). There are two major species present, each with a linker, based

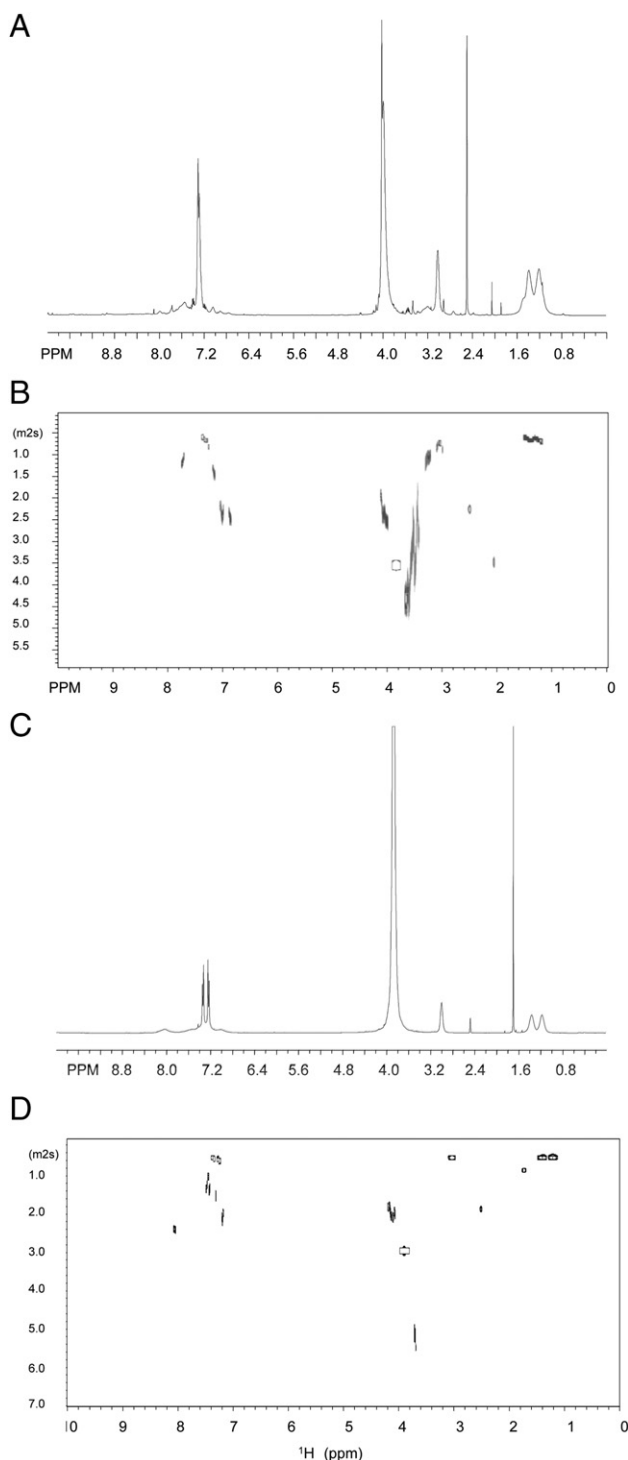


Figure 2. NMR spectra for precipitate after treating CHX with NaOCl and then dissolving in DMSO. (A) 1D ^1H NMR spectrum and (B) 2D DOSY spectrum. The sample in panels A and B was spiked with undegraded CHXa, and (C) 1D ^1H NMR and (D) 2D DOSY spectra were again collected.

on the observation of cross peaks with N-H chemical shifts, with connectivity to C-H chemical shifts. The corresponding HMQC spectrum (Fig. 3B) confirms there are 2 different parasubstituted benzene systems present (PCGH and PCU). Interestingly, the DOSY spectrum indicates that the two aromatic signals around 7.4 ppm are from undegraded CHX, whereas the pair around 7 ppm are from a fast diffusing

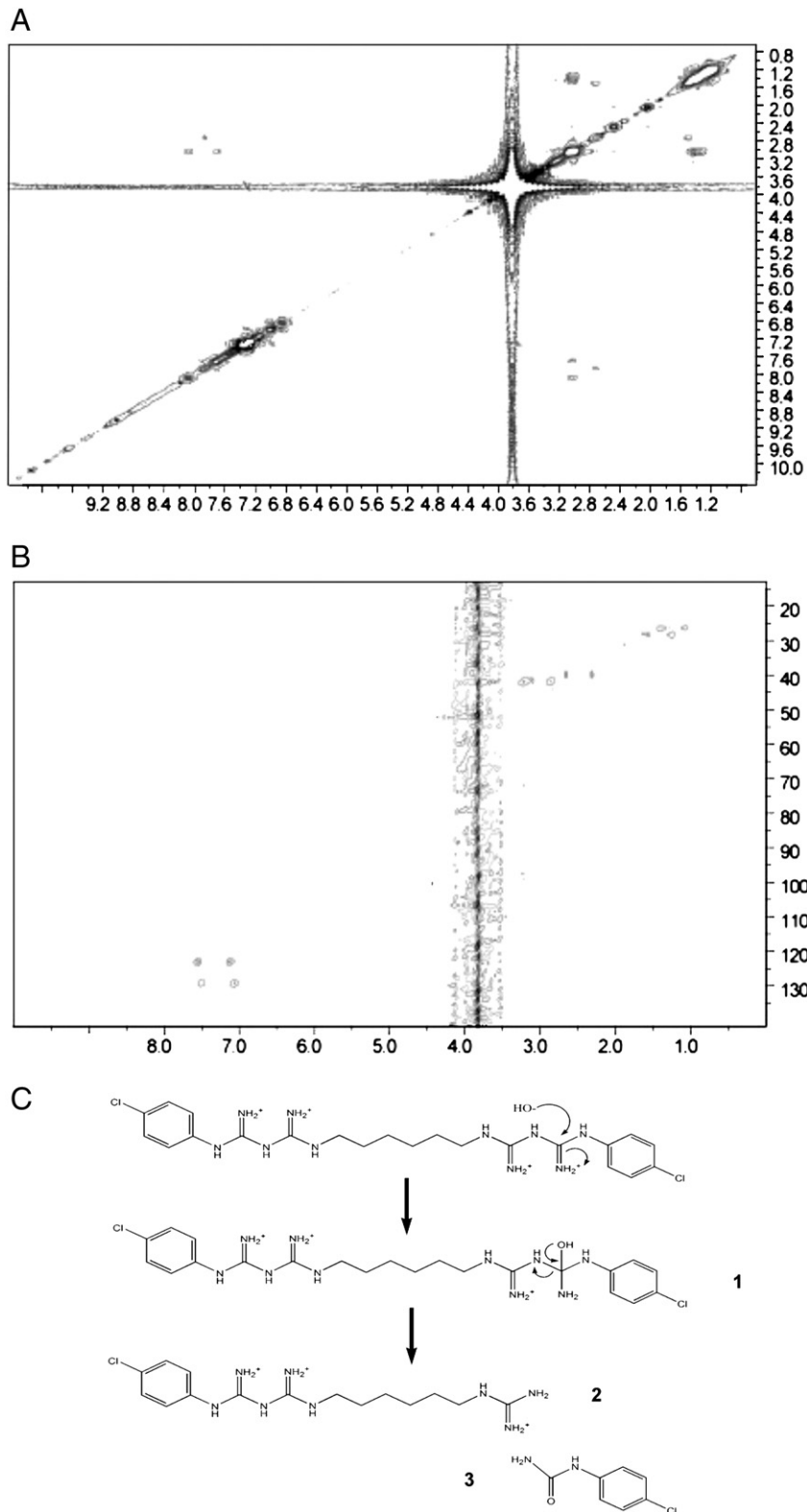


Figure 3. (A) COSY spectrum of CHX precipitate (after treatment with NaOCl) and then dissolving in DMSO. The three cross peaks at 7 to 8 ppm indicate there are three different chemical species present that have a guanido N-H that is chemically attached to a linker carbon (which has a chemical shift of 3.0). (B) HMQC spectrum of CHX precipitate (after treatment with NaOCl) and then dissolving in DMSO. In comparing the spectrum in A, note that HMQC spectra do not show N-attached protons; only C-H connectivities are observed. The two pairs of cross peaks at 7.0 and 7.5 ppm indicated there are two different substituted benzene species present. The lack of a COSY cross peak at 7.0/7.5 ppm (A) suggests that the pair at 7.0 is for one chemical species and the pair at 7.5 ppm is for the other. (C) The proposed mechanism for base-catalyzed cleavage of CHX showing tetrahedral intermediate (1) and breakdown products as PCGH (2) and PCU (3).

species (low molecular weight) that lacks any linker protons but has chemical shifts distinct from parachloroaniline (assigned to be PCU). The COSY and HMQC spectra (Fig. 3) are also consistent with the cross peaks at 7.4 being from one of the substituted benzenes, with those at 7.0 ppm being from another substituted benzene species.

The NMR spectral analysis clearly indicates that there are two breakdown products that are parachlorosubstituted benzenes but are not PCA. Based on the analysis and a reasonable chemical mechanism for the breakdown of CHX (Fig. 3C), chemical structures for the breakdown products can best be represented by the structures shown in Figure 3C referred to as PCU and PCGH.

Discussion

Previous studies examining the precipitate formed by mixing CHX and NaOCl have focused on whether the toxin PCA is produced. Basrani et al (17, 21) argued that PCA was in fact a component of the precipitate, based on mass spectrometry data. However, Thomas and Sem (20) did not observe PCA using an alternative technique, NMR. Thomas and Sem had argued that mass spectrometry might not be a reliable method for determining the presence of degradation products because it relies on gas phase ionization, which can fragment molecules. In contrast, NMR spectroscopy analyzes molecules in a noninvasive and nondestructive manner.

Krishnamurthy and Sundhakaran (18) reported that PCA was present based on Beilstein and HCl solubility tests along with NMR spectroscopy, but those tests do not identify exact breakdown products (eg, the Beilstein test only indicates that a halogen, such as chlorine, is present), and the NMR spectrum they show indicates two doublets, indicating only the presence of a parasubstituted benzene. Those observations are also consistent with the results of this study, but they do not prove that PCA was produced. Indeed, PCU and PCGH (Fig. 3C) contain chlorine and are parasubstituted benzenes, which is consistent with the data of Krishnamurthy and Sundhakaran (18) and the conclusion of Thomas and Sem (20) that PCA is not produced.

By using NMR spectroscopy, this study further examined the precipitate formed by CHX and NaOCl in order to determine a chemical composition of the breakdown products. Along with native CHX, two distinct products were recognized: a smaller breakdown product (Fig. 2B and D) that is a parasubstituted benzene that is not PCA and possesses an aliphatic linker (PCGH) and an even smaller product (PCU) that is also a parasubstituted benzene but contains no linker (Fig. 2B and D). There also appears to be residual unreacted CHX (Fig. 2B and D), and COSY and HMQC spectra clearly show two chemical species that contain a parasubstituted benzene with linker still attached, one of which is degraded (ie, PCU) and one of which is undegraded CHX (Fig. 3).

The most likely mechanism by which CHX could be degraded to produce two lower-molecular-weight species would be base-catalyzed cleavage involving nucleophilic attack at the guanido carbon to form the tetrahedral intermediate shown in Figure 3C. Breakdown of this intermediate would produce two smaller products, one with the aliphatic linker (PCGH) and one without (PCU), and both with parasubstituted benzene-like molecules that are not PCA. The resulting structures (Fig. 3C) are consistent with the DOSY and other spectra. One of these proposed products (PCU) is similar to chloroguanidine. Chloroguanidine was previously studied as a possible antimalarial drug and was found to have acute toxicity and reproductive effects in various animal models (22). Although the breakdown products identified (PCU and PCGH) are parasubstituted, the metasubstituted version of chlorophenylguanidine (*N*-[3-chlorophenyl]guanidine) is a 5-HT₃ receptor agonist and is able to cross

the blood brain barrier (23). 5-HT₃ receptor agonists were being examined for the treatment of abuse of various stimulants and emesis from chemotherapy.

This study examined the breakdown products formed by mixing NaOCl and CHX. Although the toxin PCA is not produced, a different parasubstituted molecule, PCU, and a related molecule with an aliphatic linker still attached (PCGH) is produced. Although there are no toxicology data on these compounds, literature on related compounds suggests there could be toxicity issues, and it is known that PCU may be metabolized to PCA (24, 25). Accordingly, formation of the precipitate should still be avoided by using an intermediate flush in order to prevent occluding of dentinal tubules and compromised seal of the obturated root canal. Further research should be conducted to determine the possible effects of PCU and any metabolites on dental and periapical tissues and to assess any acute or chronic toxicity.

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

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