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An *In Vitro* Spectroscopic Analysis to Determine Whether para-Chloroaniline is Produced from Mixing Sodium Hypochlorite and Chlorhexidine

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Abstract

Introduction: The purpose of this *in vitro* study was to determine whether para-chloroaniline (PCA) is formed through the reaction of mixing sodium hypochlorite (NaOCl) and chlorhexidine (CHX).

Methods: Initially commercially available samples of chlorhexidine acetate (CHXa) and PCA were analyzed with ¹H NMR spectroscopy. Two solutions, NaOCl and CHXa, were warmed to 37°C and when mixed they produced a brown precipitate. This precipitate was separated in half and pure PCA was added to one of the samples for comparison before they were each analyzed with ¹H NMR spectroscopy.

Results: The peaks in the ^1H NMR spectra of CHXa and PCA were assigned to specific protons of the molecules, and the location of the aromatic peaks in the PCA spectrum defined the PCA doublet region. While the spectrum of the precipitate alone resulted in a complex combination of peaks, upon magnification there were no peaks in the PCA doublet region which were intense enough to be quantified. In the spectrum of the precipitate, to which PCA was added, two peaks do appear in the PCA doublet region. Comparing this spectrum to that of precipitate alone, the peaks in the PCA doublet region are not visible prior to the addition of PCA.

Conclusions: Based on this *in vitro* study, the reaction mixture of NaOCl and CHXa does not produce PCA at any measurable quantity and further investigation is needed to determine the chemical composition of the brown precipitate.

Keywords: Sodium hypochlorite, chlorhexidine, para-chloroaniline, nuclear magnetic resonance (NMR)

Introduction

The goal of root canal therapy is to remove inflamed or necrotic pulp tissue from within the root canal system through both chemical and mechanical debridement. The chemical debridement can take the form of intracanal medicaments, irrigants, or lubricants which facilitate the removal of the organic and inorganic components within the root canal system. Antimicrobial rinses are used to decrease the microbial loads within the system prior to obturation and reduce the potential for failure in the future.

Sodium hypochlorite (NaOCl) is an endodontic irrigant used at varied concentrations and has been shown to have broad spectrum antimicrobial action and tissue dissolving properties ([1](#), [2](#)). While effective against microorganisms, NaOCl has also been shown to have cytotoxic effects which can cause the irritation and necrosis of periapical tissues ([3](#), [4](#)). It was also suggested by Oncag that NaOCl lacks antimicrobial substantivity in root dentin ([5](#)). These possible shortcomings of NaOCl have led researchers and clinicians to explore additional irrigants which would complement or replace NaOCl.

Chlorhexidine (CHX) ([Fig. 1A](#)) has been used in many ionic forms (acetate, gluconate, and hydrochlorate) as an intraoral

antimicrobial (6). Compared to NaOCl, CHX has been shown to be as efficacious against endodontic microbes and it lacks the cytotoxic effects of NaOCl (7, 8). CHX has also been shown to have antimicrobial substantivity in root dentin for up to 12 weeks (9, 10, 11). A possible disadvantage to CHX is that it lacks the tissue dissolving properties of NaOCl. The use of CHX in sequence with NaOCl, can possibly offer antimicrobial substantivity and tissue dissolution, respectively (12, 13). The mixture of NaOCl and CHX, however, produces a precipitate that stains the walls of the pulp chamber and has been reported to be difficult to remove (14). In addition, previous researchers have reported that this precipitate contains para-chloroaniline (PCA) (Fig. 1B), a toxin which can produce methemoglobin and is possibly carcinogenic over time (15, 16, 17).

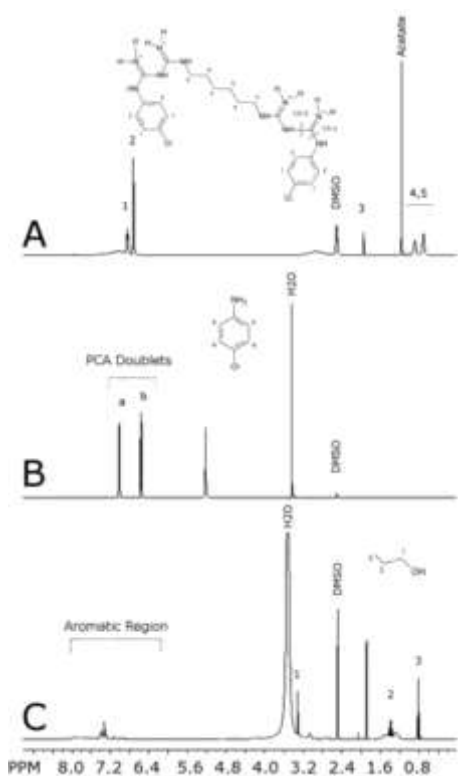


Figure 1 ¹H NMR spectrum of (A) chlorhexidine acetate with characteristic peaks at 6.85 ppm and 6.71 ppm (labels 1–5 correspond to labeled chemical structure of chlorhexidine and cleavage sites are labeled CS-1 and CS-2) (B) p-chloroaniline with characteristic peaks at 7.01 ppm and 6.56 ppm (labels a and b correspond to labeled chemical structure of PCA). (C) Reaction precipitate sampled at 60 minutes with n-propanol added at 0.4 mg/ml as an internal standard (labels 1–3 corresponding to labeled chemical structure of n-propanol). All spectra were taken with 400-MHz Varian NMR System at 25°C, acquiring 32 scans, in d₆-DMSO solvent.

Nuclear Magnetic Resonance (NMR) spectroscopy facilitates structural determinations by exposing a sample to a strong magnetic field and then to radiofrequencies which act upon atomic nuclei. Spin-active nuclei have specific spins which are acted upon by this radiation, creating a transition between energy levels which can be measured ([18](#)). This energy change is characteristic for each nucleus in a molecule, and this method of spectroscopy uses energy levels so low that even the most fragile chemical bonds remain intact. Following a chemical reaction, the presence or absence of specific molecules in a mixture can be determined by comparing the mixture's spectra to the spectra of pure compounds. The quantity of compounds present can also be determined by introducing an internal standard of a known concentration into the sample being analyzed.

The purpose of this *in vitro* study was to use Nuclear Magnetic Resonance (NMR) spectroscopy to determine whether para-chloroaniline (PCA) is formed through the reaction of mixing sodium hypochlorite and chlorhexidine.

Material and Methods

A commercially available sample of chlorhexidine acetate (CHXa) (Fischer Scientific, Pittsburgh, PA) and p-chloroaniline (Aldrich Chemical, St. Louis, MO) were analyzed with ^1H NMR spectroscopy (400-MHz Varian NMR System acquiring 32 scans/spectrum) with perdeuterated DMSO (d_6 -DMSO) as a solvent.

A 2% (0.32 M) aqueous solution of CHXa was prepared by dissolving 1.0 g solid CHXa in 50 ml of deionized H_2O . This solution was warmed to 37°C and mixed with 50 ml of 5.25 % NaOCl and stirred continuously. The brown precipitate formed immediately and a 2.0 ml sample was taken from the reaction at 60 minutes. This sample was divided in half and placed in two 1.5 ml microfuge tubes and centrifuged at 14,000 rpm for 2.5 minutes. The precipitate solid (37.6 mg) was removed from the supernatant liquid and dissolved in 1.0 ml of d_6 -DMSO. An internal standard of 0.5 μl of neat 1-propanol was added to each sample as an internal standard for ^1H NMR experiments, resulting in a 0.4 mg/ml concentration. This solution was then divided in half, and 0.1 mg/ml of pure PCA was added to one of the two

samples and ^1H NMR spectra were collected for each of the samples at 25°C.

Results

The ^1H NMR spectra were assigned for the aromatic ring protons of CHXa with peaks at 6.71 ppm and at 6.85 ppm ([Fig. 1A](#)) each exhibiting characteristic “doublet” patterns, and corresponding to protons in position 1 and 2 on the structure of CHX. A peak at 2.10 ppm corresponds to the methylene protons adjacent to the guanidine nitrogen (position 3 in [Fig. 1A](#)), and the 2 peaks at 1.38 ppm and 0.80 ppm correspond to the additional 8 methylene protons in the hexane diamine linker (positions 4 and 5 in [Fig. 1A](#)).

The ^1H NMR spectrum for PCA was also assigned, identifying peaks at 6.56 ppm and at 7.01 ppm, which comprise what is hereinafter referred to as the PCA doublet region ([Fig. 1B](#)). These peaks also each had a characteristic “doublet” splitting, and correspond to the protons labeled a and b on the structure of PCA in [Figure 1B](#).

The NMR spectra of the isolated precipitate samples were analyzed, and a complex series of peaks were found in the 6.5–8.0 ppm region ([Fig. 1C](#) and [Fig. 2A](#)). The characteristic peaks in the PCA doublet region were not present in this spectrum, although the presence of a pair of doublets in [Figure 2A](#) indicates some type of p-substituted benzene. There was also no growth of the expected PCA doublet peaks over time, which indicated that PCA was not present and was not being produced in this reaction ([Fig. 1C](#)). It was confirmed that PCA should give a signal in the expected PCA doublet region under these conditions by spiking 0.1 mg/ml of pure PCA into the sample ([Fig. 2B](#)). The characteristic peaks for PCA are labeled in [Figure 2B](#) and it is clear they were not present in the spectrum before the PCA spike ([Fig. 2A](#)).

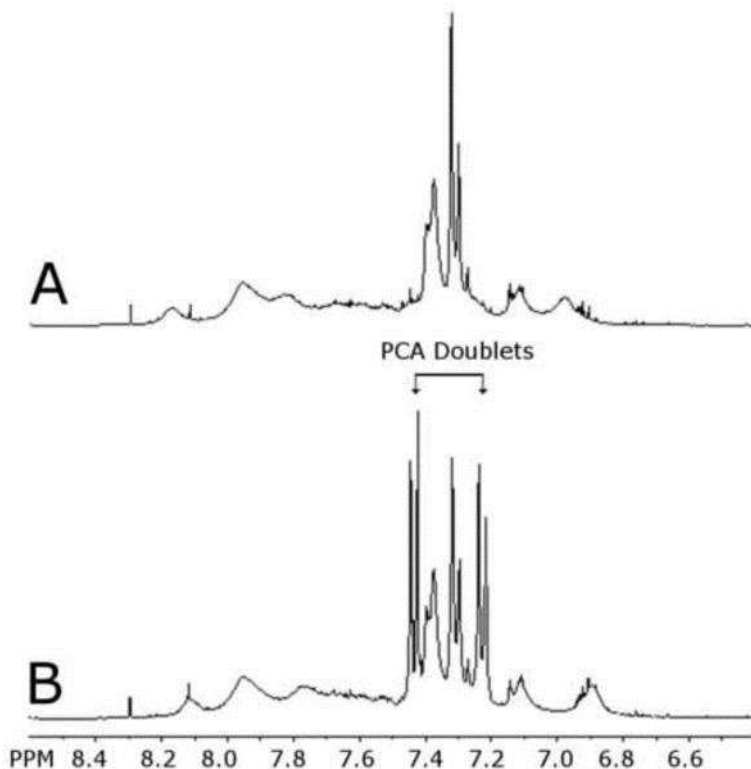


Figure 2 (A) Expanded aromatic region of the ^1H NMR spectrum of precipitate sampled at 60 minutes. Characteristic peaks in the PCA doublet region are not present. (B) Expanded aromatic region of the ^1H NMR spectrum of the precipitate sampled at 60 minutes with 0.1 mg PCA added. Characteristic peaks for PCA in the PCA doublet region are labeled. Spectra were taken with 400-MHz Varian NMR System at 25°C, acquiring 32 scans, in d_6 -DMSO solvent.

Discussion

This study analyzed the mixture of products formed by the reaction of mixing a chlorhexidine salt (CHX acetate) and NaOCl using NMR spectroscopy to determine whether PCA was produced. It has been previously shown that the degradation of CHX occurs through the cleavage of the molecule along the hexane diamine linker between the aromatic rings at either end of the molecule (19, 20). Such cleavage results in smaller degradation products, each of which contains an aromatic ring. Over time one possible degradation product could result in the formation of PCA, if the chain is separated from the aromatic ring at cleavage site-1 (CS-1) in Figure 1A. If PCA is produced in the reaction of CHX and NaOCl, the two peaks in the PCA doublet region should also show up in the spectra of the precipitate and appear as a

doublet pattern, but this is not what was observed. While the ^1H NMR spectrum of the reaction mixture resulted in a complex combination of peaks from 6.5–8.0 ppm (where peaks from aromatic compounds appear), upon magnification it is clear that there are no peaks in the PCA doublet region which are intense enough to be quantified. Only upon adding pure PCA to the 60 minute reaction sample do two peaks appear in the PCA doublet region. Comparing this spectrum ([Fig. 2B](#)) to the original 60 minute spectrum ([Fig. 2A](#)) demonstrates that the peaks in the PCA doublet region are not present prior to the addition of PCA. These data clearly demonstrate that this breakdown product is produced at levels far less than the 0.1 mg/ml of PCA that was added to the sample. This would mean that in a worst case scenario <0.5 % of chlorhexidine is decomposed to PCA. Although the pair of doublets in [Figure 2A](#) do indicate some type of p-substituted benzene is produced, it is not PCA.

This finding is in conflict with Basrani, which reported that PCA was found in the precipitate formed through the interaction of CHX and NaOCl ([15](#)). Caution was suggested when using NaOCl and CHX in a final rinse because PCA is a component of the precipitate, but assessing the risk of exposure of patients to PCA during root canal therapy had not been explored ([15](#)). This previous research used a form of mass-spectrometry which relies on gas phase ionization that can fragment molecules. This technique is, therefore, not a conclusive method for determining the presence of degradation products. There is no way to determine whether the CHX molecule was broken through chemical degradation or through the mass spectrometry process itself. NMR spectroscopy analyzes molecules present in a sample at the time the spectrum is taken in a noninvasive and nondestructive manner. If a molecule is present in solution, then the spectrum of a mixture of molecules can be compared with a standard sample, and the resulting peaks will appear with the same pattern (singlet, doublet, triplet etc.) at the same chemical shift. If a peak does not share the same pattern and chemical shift, the corresponding molecule is not present in the mixture. The absence of the expected molecule can be confirmed by adding back the pure form of the expected molecule and comparing the spectra, as in [Figure 2](#).

This study indicates that a number of breakdown products are produced when NaOCl and CHXa are mixed. These breakdown

products contain an aromatic ring that is related to PCA and is para-substituted. Based on previous studies with amine-containing molecules, it can be predicted that chlorination from NaOCl should occur at one or more of the nitrogen atoms in CHX, producing chloramines (N-Cl bonds) (21, 22). Therefore, it is not surprising that there is no cleavage at CS-1 (Fig. 1A) to produce PCA, since the aniline amine is not as good of a leaving group as the guanidinium group would be ($pK_a=14$) if cleavage occurred at CS-2 (Fig. 1A) (23). Based on this *in vitro* study, the reaction mixture of NaOCl and CHXa does not produce PCA at any measurable quantity. Further research should be conducted to determine the chemical composition of the mixture of products in the brown precipitate and their effects on teeth and periapical tissues.

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Footnotes

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