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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 relative molecular weight of the components.

Methods

Using commercially available chlorhexidine gluconate (CHXg), 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 chlorhexidine diacetate (CHXa) was then analyzed using 1D and 2D NMR spectroscopy.

Results

The 1D and 2D NMR spectra were fully assigned, in terms of chemical shifts of all proton and carbon atoms in intact CHX. This permitted identification of CHX breakdown products with and without the aliphatic linker present, including lower molecular weight components of CHX that contained a para-substituted benzene that was not para-chloroaniline (PCA).

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, neither of which are PCA.

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

Introduction

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.

Due to it's 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 (CHX) 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, 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. Kuruvilla (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 para-chloroaniline (PCA) (17–19). However, a recent study by Thomas showed that PCA was not produced at any measurable quantity (20).

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 (ex. from a proton through it's carbon, to the adjacent carbon, then to that carbon's proton). When dealing with mixtures of compounds, another two dimensional 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 assignment of carbon chemical shifts, by correlating ^1H and ^{13}C chemical shifts.

While 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, and relative molecular weight of the components.

Material and Methods

A commercially available sample of chlorhexidine gluconate (CHXg) (Sigma-Aldrich, St. Louis, MO) was analyzed with ^{1}H 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. 100μL of 0.5g/mL pure chlorhexidine diacetate (CHXa) dissolved in d_6-DMSO was added to one sample. 1D (^{1}H, ^{13}C) and 2D (COSY, DOSY, HMQC) NMR spectra were then collected for each of the samples at 25°C.

Results

Before analysis of CHX breakdown products can be undertaken, full NMR characterization of pure CHX (as the acetate salt) was performed, as summarized in Fig. 1 with assignments in Table 1. A prominent COSY cross peak in Fig. 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 $-CH_2-$ protons at position **5** as assigned to the most upfield 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 ^{1}H-^{13}C HMQC spectrum (not shown). The DOSY spectrum in Fig. 1D shows all expected peaks for protons **1**–**5**, for a molecule diffusing with a diffusion coefficient of $< 0.5 \times 10^{-10}$ m^2/sec.
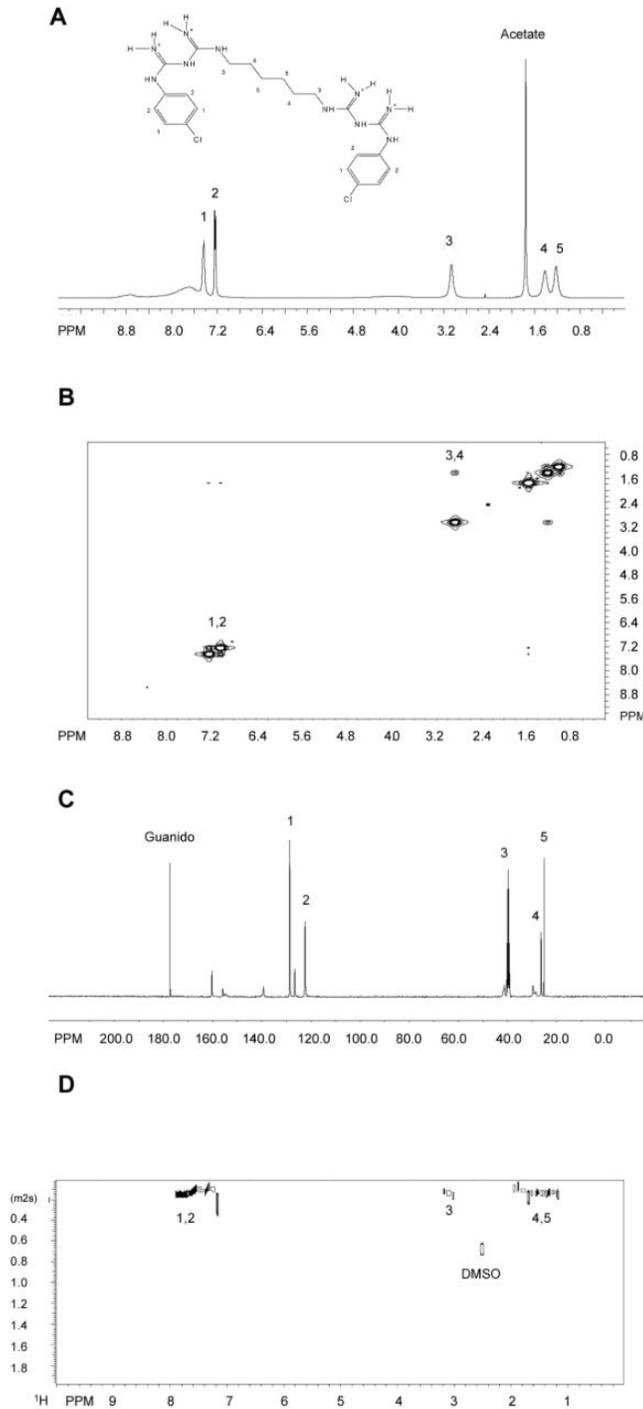
Figure 1 NMR spectra for pure CHX diacetate. (*A*) 1H NMR spectrum of CHX, with protons assigned according to the numbering scheme shown on the chemical structure of CHX, in the insert. (*B*) 2D 1H-1H COSY spectrum, showing cross peaks that were

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 ^{1}H-^{13}C HMQC spectra. (*D*) 2D DOSY spectrum, showing that only one species is present and diffusing with a diffusion coefficient of < 0.5 x 10-10 m2/sec.

Position	Proton (ppm)	Carbon (ppm)
1	7.45	129.3
2	7.24	129.1
3	3.08	41.7
4	1.43	28.9
5	1.24	26.5
Guanido (C=N)		178

Table 1 Chemical shift assignments for CHX acetate in DMSO

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, since they have larger diffusion coefficients of around $1–2.5$ x 10^{-10} m^2/sec (Fig. 2B). These breakdown products have aromatic signals around 7–8 ppm, and one also has protons with chemical shifts of 4 ppm and 2.5 ppm. In earlier NMR studies, the breakdown product was reported to be related to but distinct from para-chloroaniline (20). These chemical shifts are consistent with at least one of the breakdown products still possessing the aliphatic linker. The other breakdown product seems to be lacking the aliphatic protons, consistent with the linker being absent. The DOSY spectrum also shows the three characteristic NMR resonances for undegraded CHX, at the top of Fig. 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.
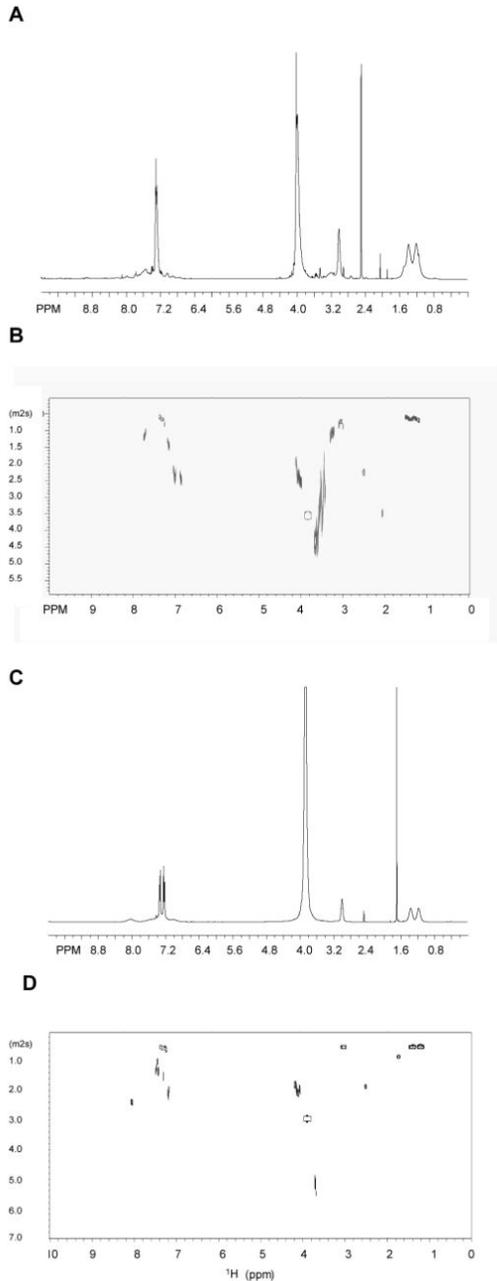
Figure 2 NMR spectra for precipitate after treating CHX with NaOCl, and dissolving in DMSO. (*A*) 1D 1H NMR spectrum, and (*B*) 2D DOSY spectrum. The sample in panels A and B was spiked with undegraded CHXa, and (*C*) 1D 1H NMR and (*D*) 2D DOSY spectra were again collected.

In a repeat of the CHX + NaOCl (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). That is, there are two major breakdown species present, each with a linker, based on the observation of cross peaks with NH chemical shifts, with connectivity to CH chemical shifts. The corresponding HMQC spectrum (Fig. 3B) confirms there are two different para-substituted benzene systems present. Interestingly, the DOSY spectrum indicates that the two aromatic signals around 7.4 ppm are from undegraded CHX, while the pair around 7 ppm are from a fast diffusing species (low molecular weight) that lacks any linker protons, but has chemical shifts distinct from para-chloroaniline. The COSY and HMQC spectra (Fig. 3) are also consistent with the cross peaks at 7.4 being from a one substitute benzene system, with those at 7.0 ppm as being from another species.
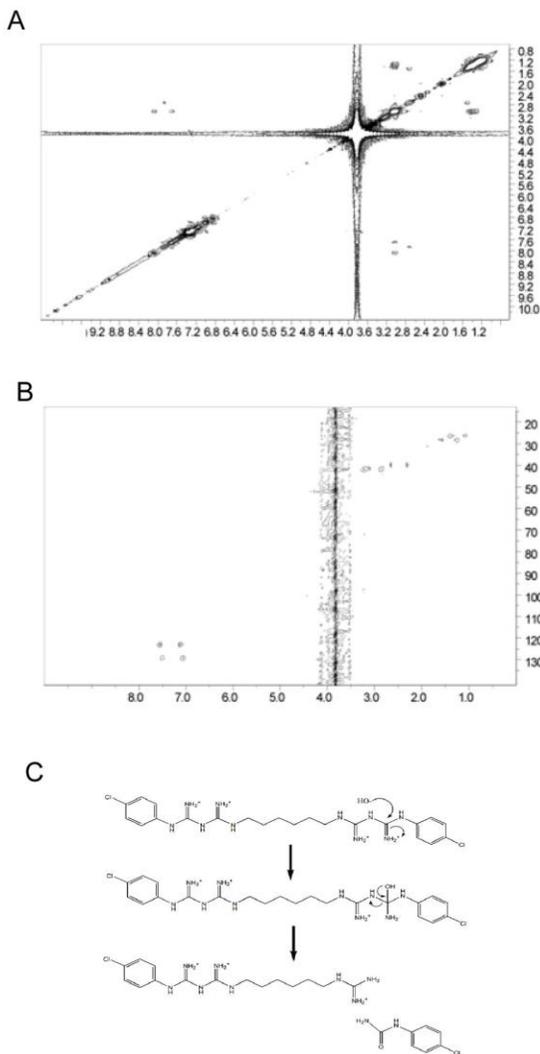
A

B

C

Figure 3 (*A*) COSY spectrum of CHX precipitate (after treatment with NaOCl), then dissolving in DMSO. The three cross peaks at around 8 ppm indicate there are three different chemical species present, which have a guanido NH that is chemically attached to a linker carbon (which has chemical shift of 3.0). (*B*) HMQC spectrum of CHX precipitate (after treatment with NaOCl), then dissolving in DMSO. In comparing with spectrum in panel 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 suggests that the pair at 7.0 is for one chemical species and the pair at 7.5 ppm is for the other. (*C*) Proposed mechanism for base catalyzed cleavage of CHX.

Discussion

Previous studies examining the precipitate formed by mixing CHX and NaOCl have focused on whether the toxin PCA is produced. Papers by Basrani et al (17,21) and Krishnamurthy et al (18) argued that PCA was in fact a component of the precipitate, based on mass spectrometry data. However, Thomas et al (20) did not observe PCA using an alternative technique, NMR. Thomas 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.

By again utilizing 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 (Figs. 2B, D) that is a para-substituted benzene that is not PCA and possesses an aliphatic linker, and an even smaller product that is also a para-substituted benzene but contains no linker (Figs. 2B, D). There also appears to be residual unreacted CHX (Fig. 2B, D), and COSY and HMQC spectra clearly show two chemical species that contain a para-substituted benzene with linker still attached, one of which is degraded and one of which is undegraded CHX (Fig. 3).

The most likely mechanism (Fig. 3C) 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 a tetrahedral intermediate. Breakdown of this intermediate would produce two smaller products, one with the aliphatic linker and one without, and both with para substituted benzene like molecules that are not PCA. The resulting structures, drawn in Fig. 3C, are consistent with the DOSY and other spectra. One of these proposed products (chlorophenylurea) 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). While the breakdown product that we have identified is para-substituted, the meta-substituted version of chlorophenylguanidine (*N*-(3-Chlorophenyl)guanidine) is a 5-HT$_3$ receptor agonist, and is able to cross the blood brain barrier (23). 5-HT$_3$ receptor agonists were being examined for the treatment of abuse of various stimulants and emesis from chemotherapy.

This study further examined the breakdown products formed by mixing NaOCl and CHX. While the toxin PCA is not produced, a different para-substituted molecule, chlorophenylurea, and a related molecule with an aliphatic linker still attached, is produced. While there are no toxicology data on these compounds, literature on related compounds suggests there could be toxicity issues, and chlorophenylurea 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 chlorophenylurea and any metabolites on dental and periapical tissues.

Acknowledgments

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