Detection of Para-Chloroaniline, Reactive Oxygen Species, and 1-Chloro-4-Nitrobenzene in High Concentrations of Chlorhexidine and in a Mixture of Chlorhexidine and Calcium Hydroxide

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Abstract

Introduction: Chlorhexidine (CHX) is likely to decompose into reactive by-products. This study evaluated the generation of 4-chloroaniline (pCA), reactive oxygen species (ROS), and 1-chloro-4-nitrobenzene in high concentrations of CHX and in a mixture of CHX and calcium hydroxide at different time points. Methods: A gas chromatography method was developed to detect pCA and CHX by-products. Mass spectroscopy was used to elucidate the structure of compounds. The samples, which were kept at 36.5°C and 95% relative humidity during the study, were analyzed immediately and 7 days after preparation. Results: pCA was detected in the 2% CHX solution and in the mixture of CHX and calcium hydroxide at all time points. pCA concentrations increased after storing under those conditions. The 2% CHX solution alone and the mixture of CHX and calcium hydroxide released ROS at all time points, but 1-chloro-4-nitrobenzene was not found. Conclusions: pCA and ROS were identified as by-products of the 2% CHX aqueous solution alone and as ointment base of calcium hydroxide paste. (J Endod 2013;39:1–5)

Key Words
Calcium hydroxide, chlorhexidine, p-chloroaniline, 1-chloro-4-nitrobenzene, reactive oxygen species

Various antimicrobial strategies have been recommended to eliminate microorganisms from the root canal system, including root canal instrumentation and the use of different irrigants, three-dimensional intracanal dressings, and coronal seals (1–4). The success of endodontic infection treatment is directly associated with the control of microorganisms and the disruption of the bacterial biofilm (5–7).

The best choice of irrigants and the precise definition of their concentrations remain challenges in endodontology. Sodium hypochlorite, chlorhexidine (CHX), and calcium hydroxide, evaluated in a large number of studies, are antimicrobial agents often used for root canal irrigation or intracanal dressing during endodontic therapy (1–7). These agents have different chemical characteristics and trigger different tissue responses. The effectiveness of their antimicrobial action varies, depending on the experimental models of endodontic infection: in vivo infected human teeth, in vitro infected canine teeth, in vitro infected human teeth, in vitro infected bovine teeth, agar diffusion tests, direct contact tests including tests with planktonic bacteria, and biofilm models (1, 3–5, 7–11).

CHX has often been recommended as an endodontic irrigant in concentrations of 0.2%–2% (5, 12–16) or in combination with calcium hydroxide (14, 15). CHX, also called di-D-glucurate or D-gluconic acid, is a compound with n, n-bis(4-chlorophenyl)-3,12-dimino-2,4,11,13-tetraazatetradecanediimidamide and molecular mass of 505.4 g/mol (17, 18). It has been widely used as an antiseptic agent for routine dental plaque control and reduction of endodontic microbiota during root canal irrigation (3, 10, 12, 13, 16, 19) but does not detoxify endotoxins (12). However, the structure of the CHX molecule, in addition to the high pH values caused by the presence of calcium hydroxide, poses a systemic risk because CHX is likely to decompose into reactive by-products such as 4-chloroaniline (pCA) (17, 20). CHX may release pCA and reactive oxygen species (ROS) as a function of time, alkaline environment (high pH), and heat (21–24). The International Agency for Research on Cancer classifies pCA in their B2 group “possibly carcinogen to humans,” which means that this agent is a possible human carcinogen (25). The interaction of CHX and calcium hydroxide may generate ROS (24), which significantly affects the cell wall and membrane structures of microorganisms. Warsis and Ahsan (26) reported that several carcinogens might also exert part of their effect by generating ROS during metabolism. Oxidative damage to cell DNA may lead to mutations and play an important role in the initiation and progression of multistage carcinogenesis. Tissue damage should be considered when CHX is used, particularly at higher concentrations, because of its toxic effects, which lead to necrotic changes in the epidermis, dermis, and subcutaneous tissue, as well as to reactive inflammatory responses (22, 27).

This study evaluated the generation of pCA and other derivatives, such as ROS and 1-chloro-4-nitrobenzene (pCNO), in CHX at a high concentration and in a mixture of CHX and calcium hydroxide at different time points.
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Material and Methods

Chemical Reagents

The research-grade chemical reagents used in this study were 20% chlorhexidine digluconate aqueous solution (product number C9394-25ml, CAS number 18472-51-0 and batch 0001440607; Sigma-Aldrich Corp, Steinheim am Albuch, BW, Germany), ≥95% calcium hydroxide, A. C. S. reagent (product number 239232 - 100g, CAS number 1305-62-0 and batch 22602JO-433; Sigma-Aldrich Corp), and ≥99% sublimed 4-chloroaniline (product number 47722-1G, CAS number 106-47-8 and batch 69966L; Sigma-Aldrich Corp). Other research-grade chemicals and reagents were also used or analyzed: acetonitrile (Solusorb; J. T. Baker, Phillipsburg, NJ), 98% 4-chloroaniline (Acros Organics, Fair Lawn, NJ), and 99% 1-chloro-4-nitrobenzene (Acros Organics).

Sample Preparation

The substances were diluted in deionized water purified by reverse osmosis by using germicide ultraviolet irradiation and 5.0-μm filters (Q842; Quimis Industria Diadema, São Paulo, Brazil). The pCA solution was prepared by dilution of 2.5 mg/mL pCA in 1.0 mL deionized water (1.96 × 10⁻² mol × L⁻¹). The 2% CHX solution was prepared by dilution of 200 μL 20% CHX in 2.0 mL deionized water. The paste was prepared by mixing 116 mg Ca(OH)₂ and 150 μL 2% CHX.

A gas chromatography/mass spectrometry (GC/MS) method was performed by using a QP2010 mass spectrometer fitted with a GC17A gas chromatograph (Shimadzu Scientific Instruments, Tokyo, Japan). Ionization voltage was 70 eV. GC was conducted in the temperature-programming mode with a DB-5MS column (50 m × 0.25 mm × 0.25 μm). First, the column temperature was 120°C for 2 minutes; then it was increased linearly at a rate of 15°C × min⁻¹ to 200°C and kept at that temperature for 10 minutes. The temperature of the injection port was 225°C, and the GC/MS interface was stored at 300°C. The helium carrier gas flow rate was 1.0 mL/min⁻¹.

A GC method was developed to detect pCA and other possible CHX by-products in the high concentrations of CHX used in clinical endodontics (2% in aqueous solution) and in a paste produced by mixing 2% CHX solution and calcium hydroxide. MS was used to determine compound structures. The samples were analyzed immediately and 7 days after preparation. During the intervals of the experiment, the samples were kept at 36.5°C and 95% relative humidity. All assays were performed in triplicate under aseptic conditions.

GC/MS analysis, where the effluent to the GC instrument is the feed to the MS instrument, is in wide use for confirmation testing of substances. Drug testing, manufacturing quality control, and environmental testing are some typical uses. When an analyst uses the GC instrument to separate compounds before analysis with an MS instrument, a complementary relationship exists, making an effective combination for chemical analysis.

Results

GC/MS Analysis of Standard pCA Solution

GC/MS was first used to determine a standard reference to detect pCA. The chromatographic analysis of compounds in the standard pCA solution immediately after preparation showed that pCA eluted at a retention time of 7.8 minutes (Fig. 1A). The MS analysis of the peak at 7.8-minute retention of pCA, immediately after its preparation, revealed a relation between mass and load (m/z) of ionized molecules in the pCA standard, with peaks at 45, 65, and 127 m/z, which confirmed the presence of pCA (Fig. 1B).

Chromatography and MS Analysis of 2% CHX at Two Time Points

pCA detection was confirmed by chromatography of the compounds of the standard 2% CHX solution immediately after preparation, which revealed a peak at a retention time of 7.8 minutes with a magnitude of about 40,000 (Fig. 1C), and by MS, which showed peaks at 45, 65, and 127 m/z (Fig. 1D). The chromatographic analysis of compounds in the 2% CHX standard solution 7 days after preparation revealed a peak at a retention time of 7.8 minutes, with a magnitude greater than 75,000 (Fig. 1E). For 2% CHX 7 days after preparation, the peak in the chromatogram (Fig. 1F) at the 7.8-minute retention time and the MS peaks at 45, 65, and 127 m/z confirmed the presence of pCA (Fig. 1F).

The magnitude of the peak at the 7.8-minute retention time plotted on the y-axis of a chromatogram for 2% CHX immediately after preparation, which was about 40,000 (Fig. 1C), was lower than that of the peak at the 7.8-minute retention time of 2% CHX 7 days after preparation, which was larger than 75,000 (Fig. 1E). Such findings suggested that storing the 2% CHX solution at 36.5°C and 95% relative humidity for 7 days increased pCA generation.

GC/MS Analysis of Paste at Two Time Points

pCA detection was confirmed by chromatography of the compounds of a paste produced by mixing 2% CHX solution and calcium hydroxide immediately after preparation, which revealed a peak at a retention time of 7.8 minutes, with a magnitude of about 40,000 (Fig. 1G), and by MS, which showed peaks at 45, 65, and 127 m/z (Fig. 1H). Seven days after preparation, chromatography revealed a peak at the 7.8-minute retention time, with a magnitude of close to 80,000 (Fig. 1I), and MS showed peaks at 45, 65, and 127 m/z (Fig. 1J), which also confirmed the presence of pCA. The magnitude of the peak at the 7.8-minute retention time plotted on the y-axis of a chromatogram of the paste immediately after its preparation, which was about 40,000 (Fig. 1G), was lower than the magnitude of the peak at the 7.8-minute retention time of the paste 7 days after preparation, which was close to 80,000 (Fig. 1I). Such findings suggested that storing the mixture of 2% CHX solution and Ca(OH)₂ at 36.5°C and 95% relative humidity for 7 days increased pCA generation.

pCA and pCNO were observed in control by using standard solutions of these substances. Data of pCNO were not detected.

Discussion

pCA and ROS were detected in 2% CHX and in the combination of 2% CHX and calcium hydroxide at all time points, but pCNO was not found.

GC/MS was used to evaluate the production of potentially toxic factors such as pCA, which originate from high concentrations of CHX (2% aqueous solution), as well as from mixing CHX and calcium hydroxide. pCA may be analyzed by using chromatography (6, 21, 23, 24, 28). Barbin et al (24) detected pCA in solutions with low concentrations of CHX (0.2% aqueous solution) stored at 36.5°C and 95% relative humidity 14 days after preparation, but no traces of pCA were found in the paste produced by mixing 0.2% CHX and calcium hydroxide at different time points, although ROS were generated by 0.2% CHX alone and CHX mixed with calcium hydroxide at all time points. Those authors also found that there were no traces of CHX in the paste (0.2% CHX + calcium hydroxide) immediately and 7 and 14 days after preparation when stored at 36.5°C and 95% relative humidity. This finding suggests that the addition of calcium hydroxide to 0.2% CHX leads to an immediate and total degradation of low-concentration CHX (0.2% aqueous solution). The correlation of those findings with the results of our study may indicate that higher CHX concentrations (2% aqueous solution) increase pCA generation in...
both substances (2% CHX alone and in combination with calcium hydroxide) immediately and 7 days after preparation.

This study detected the presence of pCA in 2% CHX and in a paste produced by mixing CHX with calcium hydroxide both immediately after preparation and after storage for 7 days at a temperature of 36.5°C and 95% relative humidity and suggests that pCA concentrations increased after storing under those conditions. Palarettia et al (29) developed a high-performance liquid chromatography method for CHX and its metabolites (pCA and pCNO) produced by oxidation reactions mediated by metallorporphyrins such as cytochrome P-450 models for metabolite prediction. They found that the presence of metallorporphyrins in the reaction substantially increased the formation of pCA (up to 58%) as

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**Figure 1.**

(A) Chromatogram of standard pCA solution immediately after preparation; elutes from column at retention time of 7.8 minutes; x-axis shows retention time in minutes, and y-axis, signal intensity in total ion current (TIC) (×1,000,000). (B) Mass spectrum of stock solution of pCA immediately after preparation; peaks at 45, 65, and 127 m/z; x-axis shows mass-to-charge ratio (m/z), and y-axis, relative abundance (percentage). (C) Chromatogram of compounds in standard 2% CHX solution immediately after preparation: peak at 7.8-minute retention time and magnitude of about 4.0 × 10,000; x-axis shows retention time in minutes, and y-axis, signal intensity in TIC (×10,000). (D) Mass spectrum of stock solution of 2% CHX immediately after preparation; peaks at 45, 65, and 127 m/z; x-axis shows mass-to-charge ratio (m/z), and y-axis, relative abundance (percentage). (E) Chromatogram of compounds in standard 2% CHX solution 7 days after preparation; peak at 7.8-minute retention time and magnitude greater than 0.75 × 100,000; x-axis shows retention time in minutes, and y-axis, signal intensity in TIC (×100,000).
a CHX metabolite, especially at a neutral pH. Their findings provided evidence of a possible in vivo formation of pCA as a CHX metabolite mediated by cytochrome P-450 metabolism, which might even increase the risks to the health of patients treated with a 2% CHX solution or a paste produced by mixing 2% CHX and calcium hydroxide. The formation of chloroaniline from all chloronitrobenzene isomers was also mediated by cytochrome P-450 metabolism. In addition, pCA is a common chemical intermediate produced from pCNO (30), which may explain why pCNO was not found in this study, because there may have been a total conversion of pCNO into pCA. Cutaneous absorption of CHX is not significant (31), which minimizes its toxicity when used topically, but the possible presence of CHX in host tissue during endodontic treatment, when CHX may extrude through the apical foramen, may lead to the spreading of CHX or its toxic by-products, such as pCA, pCNO, and ROS, in the periapical tissue and the lymphatic and circulatory systems.

Some chemical interactions may be observed between substances used in endodontics (21–24, 32–35). CHX degrades into pCA and several reactive by-products, and its substantial antimicrobial activity and residual effects have been demonstrated (19, 36, 37). Particularly when applied in clinical endodontics at high concentrations for 7 days or longer (38), it may prolong pCA generation, and both CHX and its by-products may remain in host tissues for long periods of time. The combination of these effects may increase the potential systemic risk of CHX.

pCA is a persistent environmental degradation product found in some herbicides and fungicides, has potential long-term toxicity and carcinogenicity in male rats and male mice, and may cause

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**Figure 1.** (continued) (F) Mass spectrum of stock solution of 2% CHX 7 days after preparation; peaks at 45, 65, and 127 m/z; x-axis shows mass-to-charge ratio (m/z), and y-axis, relative abundance (percentage). (G) Chromatogram of compounds in paste produced by mixing 2% CHX solution with calcium hydroxide immediately after preparation; peak at 7.8-minute retention time and magnitude of about 0.4 × 100,000; x-axis represents retention time in minutes, and y-axis, signal intensity in TIC (×100,000). (H) Mass spectrum of paste produced by mixing 2% CHX solution with calcium hydroxide immediately after preparation; peaks at 45, 65, and 127 m/z; x-axis shows mass-to-charge ratio (m/z), and y-axis, relative abundance (percentage). (I) Chromatogram of compounds in paste produced by mixing 2% CHX solution with calcium hydroxide 7 days after preparation; peak at 7.8-minute retention time and magnitude of about 0.8 × 100,000; x-axis shows retention time in minutes, and y-axis, signal intensity in TIC (×100,000). (J) Mass spectrum of paste produced by mixing 2% CHX solution with calcium hydroxide 7 days after preparation; peaks at 45, 65, and 127 m/z; x-axis shows mass-to-charge ratio (m/z), and y-axis, relative abundance (percentage).
methylene blue (21, 30). In addition, pCA induces DNA damage in bacteria. However, the results of gene mutation are still inconclusive. Gene mutation, but not mitotic recombination, has been induced in fungi, and gene mutation, sister chromatid exchange, and chromosomal aberrations have been induced in cultured mammalian cells, but conflicting data were obtained for cell transformation. Although pCA carcinogenicity in experimental animals has been demonstrated, there is not enough evidence of it in humans. However, results indicate that it may also be carcinogenic to humans, and the International Agency for Research on Cancer has listed it under group 2B “possibly carcinogen to humans” (21, 30). Kačmář et al (39) evaluated the toxic and immunotoxic effects of a pCA metabolite of a herbicide (monolinuron) in suspensions of peripheral blood leukocytes of 5 sheep by using a migration-inhibition test. The toxic effects of pCA were recorded at concentrations of 1.0–0.1 mg/mL and immunotoxic effects at 0.01–0.001 mg/mL. Results confirmed total inhibition of leukocyte migration. Immunotoxic effects, defined as mitogenetic activation of leukocytes by phytohemagglutinin, concanavalin A, and lipopolysaccharide, were detected at pCA concentrations 10–100 times lower than those that resulted in toxic effects.

The costs (systemic risks) and benefits of choosing CHX as a first-choice agent to control endodontic infection must be weighed against those of other substances with antimicrobial properties, so that the goals of endodontic treatment may be achieved predictably and without unnecessary risks of systemic damage. Further studies should evaluate which CHX concentrations are safe for use in humans by assessing how much pCA may be generated when using 0.12%–2% CHX solutions, gel, and combinations with other substances.

Acknowledgments

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

References