Using Diazotization to Characterize the Effect of Heat or Sodium Hypochlorite on 2.0% Chlorhexidine

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Abstract

Introduction: The combination of sodium hypochlorite (NaOCl) and chlorhexidine (CHX) results in the formation of a precipitate. In a previous study, we demonstrated the formation of 4-chloraniline (PCA) in the precipitate in an amount directly related to the concentration of NaOCl used. **Aims and Methods:** The aim of the present study was to use a diazotization technique to confirm the presence of an aromatic amine (like PCA) in the NaOCl/CHX precipitate and also in the 2.0% CHX at different temperatures (37°C and 45°C). **Results:** The results corroborated the presence of the aromatic amine in the precipitate and in the CHX at 45°C. **Conclusions:** Further investigations of the precipitate should address the bioavailability of PCA leaching out from dentin and its cytotoxicity. Until the precipitate is studied further, it would appear prudent to minimize its formation. *(J Endod 2009;35:1296–1299)*

Key Words

Chlorhexidine, diazotization technique, interaction of irrigants, precipitate, sodium hypochlorite

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Bacteria in the root canal system provoke the formation of periapical inflammatory lesions (1). The aim of root canal treatment is to eliminate bacteria from the infected root canal and to prevent reinfection. Biomechanical cleaning and shaping of the root canal greatly reduce the number of bacteria (2). Nevertheless, studies have shown that bacteria often persist (3). Various irrigants have been used for canal disinfection (2, 4, 5). The most common irrigant used is sodium hypochlorite (NaOCl) in concentrations ranging from 0.5%–6% (6–8). NaOCl is an effective tissue solvent and antimicrobial agent (6–8). Its germicidal ability is related to the formation of hypochlorous acid when in contact with bacteria and organic debris. In high concentration NaOCl is toxic and can cause periapical inflammation (6, 7, 9, 10), whereas in low concentrations its antimicrobial effect is reduced, especially against specific microorganisms (6). NaOCl is not a substantive antimicrobial agent (11). It also discolors fabrics on contact (9), corrodes instruments, and has an unpleasant odor (11).

Chlorhexidine gluconate (CHX) is a broad-spectrum antimicrobial agent that has been advocated for root canal disinfection (8, 12). When used as an irrigant or intracanal medication, its antibacterial efficacy is comparable to that of NaOCl (11, 13, 14), and it is effective against certain NaOCl-resistant bacterial strains (11, 15). Prolonged exposure of the root dentin to CHX might impart a residual antimicrobial property to the dentin surface (11, 14, 16, 17). CHX has a low grade of toxicity (18); however, its inability to dissolve organic matter might be a drawback in its clinical use (19).

A combination of NaOCl and CHX for root canal irrigation has been advocated to enhance their antimicrobial properties. A study by Kuruvilla and Kamath (7) suggested that the antimicrobial effect of 2.5% NaOCl and 0.2% CHX used in combination was greater than that of either agent used separately. Zehnder (20) proposed an irrigation regimen in which NaOCl would be used during root canal enlargement followed by irrigation with ethylenediaminetetraacetic acid (EDTA), and CHX as a final flush. However, when NaOCl was present in the canal at the time CHX was introduced, a precipitation was observed to occur (20, 21), as well as the combination of CHX and EDTA produces a white precipitate (22). In a previous study (23), formation of a precipitate was observed after mixing NaOCl and 2%CHX. By using x-ray photon spectroscopy (XPS) and time of flight secondary ion mass spectrometry (TOF-SIMS), we demonstrated the presence of 4-chloroaniline (PCA) in an amount directly related to the concentration of NaOCl used. Subsequently, Bui et al (24) reported that the NaOCl/CHX precipitate tended to occlude the dentinal tubules and that further investigation was needed. The presence of PCA was also reported in a recent study (25) in a 0.2% CHX solution after 14 days.

The use of ultrasonic energy to enhance the efficacy of irrigants is a new trend in clinical endodontics. It was suggested that passive ultrasonic vibration used in conjunction with NaOCl significantly improved removal of bacteria from the root canal (26). Cameron (26) reported that an increase in the intracanal temperature from 37°C to 45°C occurred close to the tip of the instrument when the NaOCl was ultrasonically activated for 30 seconds without replenishment. No studies on the effect of CHX with passive ultrasonic vibrations have been reported, and it could be suspicion that heated 2.0% CHX might form a precipitate.

Therefore, the aim of the present study was to use a diazotization technique (reaction between a primary aromatic amine and nitrous acid to give a diazo compound) to confirm the presence of an aromatic amine (like PCA) in the NaOCl/CHX precipitate and also in the 2.0% CHX at different temperatures (37° and 45°C).

Materials and Methods

Solutions

The precipitate was created by using 6.0% NaOCl (Fisher Scientific, Fair Lawn, NJ) and 2.0% CHX (Willer-PCCA, London, ON, Canada). The CHX solution was prepared by diluting 10 mL of 20% CHX in 90 mL of filter-sterilized distilled water (dH_20). The 2.0% CHX solution was used at room temperature, 37°, and 45°C.

PCA (ACP Chemicals, Montreal, Quebec, Canada) and aniline (ACP Chemicals) were used as controls.

The following solutions were prepared for the reaction of amines: 3 mol/L and 1 mol/L hydrochloric acid (HCl) (Fisher Scientific, Nepean, Ontario, Canada); 1 mol/L sodium nitrite ($NaNO_2$) (BDH Inc, Toronto, Ontario, Canada); 3 mol/L sodium carbonate ($NaCO_3$) (BDH Inc, Toronto, Ontario, Canada).

Formation of the Precipitate

A mixture of 0.5 mL of 6.0% NaOCl and 0.5 mL 2.0% CHX was prepared in a flat-top 1.5-mL polypropylene micro-tube. A precipitated brown mass was suspended at the top of the tube. The precipitation was instantaneous and showed no change with time (23). Before analysis, the precipitate within the micro-tubes was washed several times with sterile dH_2O to remove ions that could interfere with the analysis. This precipitate was kept to be used in the diazotization reaction.

Heating of CHX

Two 50-mL tubes of 2.0% CHX were prepared and kept in a water bath with a constant temperature of either 37°C or 45°C for 45 minutes. One tube of CHX was kept at room temperature.

Diazotization to Detect Aromatic Amines

The following samples were prepared: group A (control): 10 mmol of PCA; group B (control): 10 mmol of aniline; group C (control): 2.0%CHX at room temperature; group D (experimental): 2.0%CHX at 37°C; group E (experimental): 2.0%CHX at 45°C; and group F (experimental): precipitate formed when 2.0%CHX was mixed with 6.0% NaOCl. All groups were dissolved in 8 mL of 3 mol/L HCl each. All the solutions were cooled to 5°C in an ice bath, and 10 mL of freshly prepared 1 mol/L $NaNO_2$ was added to each tube while stirring slowly. The rate of addition was adjusted to keep the temperatures below 10°C. The solutions were tested with Starch Iodide paper (Fischer Scientific, Ottawa, Ontario, Canada) to test for the production of nitrous acid. The $NaNO_3$ was added drop-wise until the paper turned violet-blue.

These diazonium salt solutions were kept in an ice bath and used right away in the coupling reaction.

Coupling with the Amine Aniline

In 5 separate test tubes, 10 mmol of aniline was dissolved in 10 mL of 1 mol/L HCl each and cooled in an ice bath. The aniline is the coupling component. While stirring, each diazonium salt solution (groups A, B, C, D, E, and F) was added to the coupling component solution and kept in an ice bath for 15 minutes. Each solution was neutralized with 3 mol/L sodium carbonate until it was neutral to litmus paper. Coupling is most efficient at a neutral pH. After neutralization, the solutions were kept in an ice bath until crystallization was complete, and the color of the end product was recorded immediately.

This test was repeated 3 times to assess the reproducibility of the results.

Results

The end products of the PCA, the NaOCl/CHX precipitate (Fig. 1), and 2.0% CHX at 45°C were yellow, indicating that an aromatic amine was present in all samples.

However, CHX at room temperature or heated at 37°C (Fig. 2) turned white, indicating that no aromatic amine was present.

Discussion

This study used a technique called diazotization (27, 28) to detect the presence of an aromatic amine (like PCA) in the precipitate formed when CHX and NaOCl are mixed and also in the 2.0% CHX at different temperatures (37°C and 45°C). Diazotization is a technique used to form azo dyes, which are bright in color and contain an azo group that is 2 nitrogen atoms double bonded to each other. This technique is widely used in the fabric industry with aniline to create vibrant dyes. The color of the formed azo dye is dependent on the benzene ring present in aromatic amines. PCA is an aromatic amine with a benzene ring.

In the diazotization process, aromatic amines are treated with nitrous acid to yield diazonium salts. The nitrous acid is generated *in situ* from the sodium nitrate and a mineral acid, usually HCl, in the following reaction:

$$HCl + NaNO2 \rightarrow HNO2\ (\text{nitrous acid}) + NaCl$$

Once the salts are prepared, they are coupled with another aromatic amine, in our case aniline. Coupling is where the azo compound or dye

Figure 1. Tubes with aniline, PCA, and the NaOCl/CHX precipitate turned yellow, indicating that the amine was present.

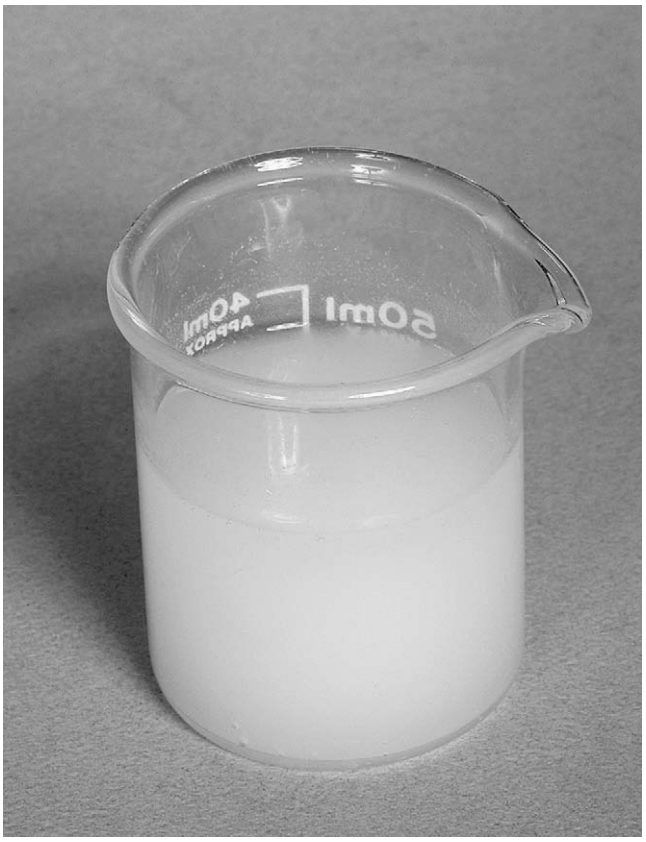
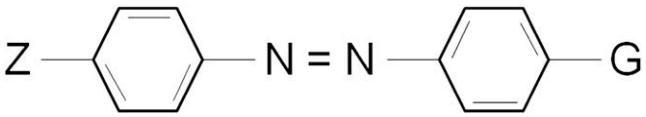


Figure 2. Tube with 2.0% CHX heated at 37°C turned white, indicating that there is no amine present.

is formed. The diazo group or salt from the diazotization process reacts or attacks another aromatic molecule. The diazo group is a weak electrophile, which means it is weakly attracted to electrons; therefore, the reaction works best when the aromatic molecule under attack contains a group that is electron rich, like the amino group in aniline (29). This is where the double nitrogen bond is formed; the reaction is as follows:



The resulting end product is a yellow azo dye when an aromatic amine such as PCA is present.

Our findings showed that when PCA was diazotized as a control, the end product was yellow, as a result of the fact that PCA is an aromatic amine. On the basis of this fact, we diazotized the precipitate formed when NaOCl and CHX are mixed; this resulted in the same yellow end product as well, indicating the presence of an aromatic amine that is in PCA. We also diazotized CHX at room temperature, at 37°C, and at 45°C to determine whether any aromatic amine is formed when CHX breaks down on heating. Our findings showed that CHX at room temperature and at 37°C did not result in a yellow end product when diazotized, therefore indicating that there is no aromatic amine present. However, when CHX that was heated to 45°C was diazotized, the result was a yellow end product, indicating the presence of PCA or another aromatic amine.

These findings might be clinically relevant because PCA has been shown to be toxic (30, 31). Toxicologic studies in animals have shown that the hematopoietic system is the major target for PCA (30). The primary toxic effect of PCA is the formation of methemoglobin. Studies have shown that methemoglobin formation and hemolytic anemia occurred in animals after 90-day exposure to PCA (31). A carcinogenic effect of PCA in rats, manifested by increased sarcomas in the spleen, was also reported. Another reported effect was in exposed zebra fish, in which eggs were hatched later and displayed increased rates of abnormal development and pigmentation (31). In humans, there have been reports of severe methemoglobinemia in neonates exposed to PCA as a result of CHX broken down to PCA by the humidifier heater in neonatal incubators (32).

Considering that CHX can break down to form PCA by exposure to heat, this study was designed to verify the formation of PCA in heated CHX. In a clinical situation, ultrasonic energy is recently advocated as a means of removal of the smear layer and bacteria from the root canal (26). Although to date no studies have shown an enhanced antibacterial effect of the combination of CHX and ultrasonic energy, it is conceivable that clinicians who use CHX might consider using vibration to enhance the distribution and effect of the irrigant within the root canal. It has been shown that ultrasonic activation might increase the temperature to at least 45°C (26). Heated CHX at 45°C displayed the possible formation of PCA; therefore, ultrasonic activation of CHX should be avoided until further investigation is done.

In conclusion, this study verified the presence of an aromatic amine (like PCA) in the precipitate formed when 6.0% NaOCl and 2.0% CHX are mixed and when 2% CHX is heated at 45°C. The presence of an aromatic amine might also be indicative of other isomers of aniline, such as 2-chloroaniline and 3-chloroaniline. Because all isomers of aniline might have possible toxic effects, it is advised that NaOCl not be used in combination with CHX or with ultrasonic activation that might raise the temperature above 37°C until more information is available.

Further investigations of the NaOCl/CHX precipitate in endodontic situations should address the bioavailability of PCA leached out of treated dentin and its cytotoxicity. In the meantime, it would appear prudent to minimize its formation by washing away and drying any remaining NaOCl with alcohol or EDTA and paper points before using CHX.

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