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Original scientific paper

INVESTIGATION OF *P*-CHLOROANILINE FORMATION IN THE REACTIONS BETWEEN DIFFERENT ENDODONTIC IRRIGANTS

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ABSTRACT. The aim of this study was to determine whether *p*-chloroaniline (PCA) is formed in the reactions of sodium hypochlorite (NaOCl) with final rinse chlorhexidine (CHX), QMix (combination of ethylenediaminetetraacetic acid (EDTA), CHX and detergent) and EDTA/CHX solutions by thin-layer chromatography (TLC), proton nuclear magnetic resonance (^{1}H NMR) and infrared (IR) spectroscopy. Commercially available 5.25% NaOCl solution was mixed with 2% CHX and QMix in 1:1 (v/v) ratio at room temperature. Furthermore, 2% CHX was associated with 17% EDTA under the same experimental conditions. The obtained solutions were evaluated qualitatively for color changing, precipitate and/or bubble formation and analyzed by TLC chromatography. The association products were investigated by spectroscopic (^{1}H NMR and IR) methods in order to determine whether they contain PCA. It was found that interactions between NaOCl/CHX and CHX/EDTA led to forming of brown and white precipitate. When NaOCl was mixed with QMix, an orange-brown precipitate was formed. PCA was not detected as a product of the reactions between NaOCl and final rinse solutions of CHX, QMix and EDTA/CHX association.

Key words: *p*-Chloroaniline, Infrared spectroscopy, Irrigants, Proton nuclear magnetic resonance, Thin-layer chromatography

INTRODUCTION

Root canal irrigation, mechanical or chemical, aims to remove debris, lubricate the canal, dissolve organic and inorganic tissues, as well as to prevent smear layer formation or aid its removal (ZEHNDER *et al.*, 2005; HÜLSMANN *et al.*, 2007; HAAPASALO *et al.*, 2014). The biological function of the irrigants is related to their antimicrobial effect, inactivation of

endotoxin, non-toxicity to vital tissues and low allergenic potential (ZEHNDER *et al.*, 2005). The most commonly used irrigants in endodontic therapy are sodium hypochlorite (NaOCl), ethylenediaminetetraacetic acid (EDTA) and chlorhexidine (CHX) (ZEHNDER *et al.*, 2005; HÜLSMANN *et al.*, 2007; GU *et al.*, 2009; BITTER *et al.*, 2013; HAAPASALO *et al.*, 2014). In order to reduce surface tension of irrigants, improve their dentinal tubules penetration and enhance their efficiency, combination of two or more irrigants is recommended (STOJICIC *et al.*, 2010). Accordingly, QMix (combination of EDTA, CHX and detergent; Dentsply TulsaDental, Tulsa, OK, USA) have been introduced in endodontic practice (STOJICIC *et al.* 2012). Previous studies showed that QMix is as effective as 17% EDTA in removal of smear layer or even superior (DAI *et al.*, 2011; STOJICIC *et al.*, 2012). Moreover, QMix is as efficacious antimicrobial agent as 6% NaOCl and superior when compared to CHX, MTAD (mixture of doxycycline, citric acid and detergent) and solutions of NaOCl with lower concentrations (DAI *et al.*, 2011; WANG *et al.*, 2012).

Numerous *in vitro* studies showed that chemical interactions between some irrigants result in formation of precipitates (TAY *et al.*, 2006; BASRANI *et al.*, 2007; RASIMICK *et al.*, 2008; ARSLAN *et al.*, 2015). Clinical relevance of the precipitate is in its difficult removal from the canal which can contribute to periapical *p*-chloroaniline (PCA) leaching (VIVAQUA-GOMES *et al.*, 2002). Some of these studies (BASRANI *et al.*, 2007; BASRANI *et al.*, 2010) demonstrated that successive rinsing of root canal with NaOCl and CHX produced an orange-brown precipitate, which contains PCA. However, these findings were not confirmed in the other studies (THOMAS *et al.*, 2010; ORHAN *et al.*, 2016). It is worth noting that PCA is carcinogenic and toxic (CHHABRA *et al.*, 1991; KACMAR *et al.*, 1995) and can lead to dentin discoloration (SOUZA *et al.*, 2013). After mixing CHX and EDTA, non-toxic white precipitate formation was noticed (RASIMICK *et al.*, 2008). There are only a few studies related to precipitate formation in the interactions of most commonly used irrigant NaOCl with QMix solution (ARSLAN *et al.*, 2015; KOLOSOWSKI *et al.*, 2014), in which PCA was not detected.

A recent study of ORHAN *et al.*, 2016 has showed that different methods can be used for PCA presence analysis in the precipitate resulted from NaOCl/CHX association. Additionally, THOMAS *et al.*, 2010 reported that mass spectrometry is not a reliable method for PCA formation detection, as well as Beilstein and HCl solubility tests (KRISBNAMURTBY *et al.*, 2010). Therefore, the aim of this study was to determine whether PCA is formed in the reactions of NaOCl with final rinse CHX and QMix solutions, as well as a result of EDTA/CHX combination by thin-layer chromatography (TLC), proton nuclear magnetic resonance (^{1}H NMR) and infrared (IR) spectroscopy.

MATERIALS AND METHODS

Chemicals

Commercially available solutions (5.25% NaOCl, 2% CHX (Consepsis, Dentsply Tulsa Dental, Tulsa, OK, USA), 17% EDTA (ENDO-SOLution, Cerkamed, PPH Cerkamed, Stalowa Wola, Poland) and QMix) were used in the study. PCA, ethyl acetate, hexane, sodium sulfate and deuterated dimethyl sulfoxide (DMSO-d_6) were purchased from the Sigma-Aldrich (St. Louis, Missouri, USA). All the employed chemicals were of analytical reagent grade and used without further purification.

Associations

5 mL of 5.25% NaOCl solution was mixed with 5 mL of 2% CHX and QMix at room temperature. In addition, 2% CHX was associated with 17% EDTA in 1:1 (v/v) ratio. The

final solutions were evaluated qualitatively for color changing, precipitate and/or bubble formation. The visual characteristics of the irrigants' resultant association were summarized in Table 1.

Table 1. Visual characteristics of the irrigants association observed in this study.

Solution 1	Solution 2	Visual characteristic of the association*
5.25% NaOCl	2% CHX	Brown precipitate
5.25% NaOCl	QMix	Orange-brown coloring and bubble formation
2% CHX	17% EDTA	White cloudy precipitate

*Solutions were mixed in 1:1 (v/v) ratio at room temperature.

PCA Formation analysis

After mixing 5.25% NaOCl with 2% CHX, a brown-colored mixture was extracted three times with 15.0 mL ethyl acetate, and then organic layer was separated and dried over anhydrous sodium sulfate (ORHAN *et al.*, 2016). This solution was analyzed by TLC and then ethyl acetate was removed under reduced pressure. The isolated brown precipitate was further evaluated by spectroscopic (^{1}H NMR and IR) methods in order to determine whether it contains PCA.

In the case of the other two irrigants associations, the resulting solution was analyzed by TLC and then solvent was removed under reduced pressure. The obtained solids (white for 2% CHX/17% EDTA and orange-brown for 5.25% NaOCl/QMix) were further analyzed by spectroscopic (^{1}H NMR and IR) methods.

Silica gel 60 on Al plates with layer thickness 0.2 mm (Merck) was used for TLC. Two drops of the corresponding solution were injected on TLC plate. Near this spot, two drops of solution obtained by dissolving 2.0 mg of PCA in 1.0 mL of ethyl acetate were injected as stationary phase. This plate was placed in a TLC tank containing ethyl acetate/hexane mixture in 1:2 (v/v) ratio as mobile phase. ^{1}H NMR spectra were recorded at 25 ^{o}C on a Varian Gemini 2000 spectrometer at 200 MHz. 20.0 mg of PCA and the corresponding association products were dissolved in 0.7 mL of DMSO-d_6 and transferred into a 5 mm NMR tube. Chemical shifts are reported in ppm (δ) and scalar couplings are reported in Hertz. IR spectra were recorded as KBr pallets on a Perkin Elmer Spectrum One spectrometer over the range 4000-450 cm^{-1}.

RESULTS AND DISCUSSION

The visual irrigants' resultant association characteristics are summarized in Table 1. Brown and white precipitates were formed in the interactions of 2% CHX with 5.25% NaOCl and 17% EDTA, respectively, immediately after mixing of these irrigants, while the combination of 5.25% NaOCl with QMix resulted in orange-brown solution coloration and bubbles formation.

Different irrigating solutions combinations were studied for PCA presence by TLC, ^{1}H NMR and IR measurements. The corresponding data for each combination were compared with those for PCA.

TLC plates under UV light for the 5.25% NaOCl/QMix association and PCA before and after corresponding solvent development are shown in Fig. 1. The same picture is

obtained for the other irrigant associations, indicating that none of them produces PCA as a by-product.

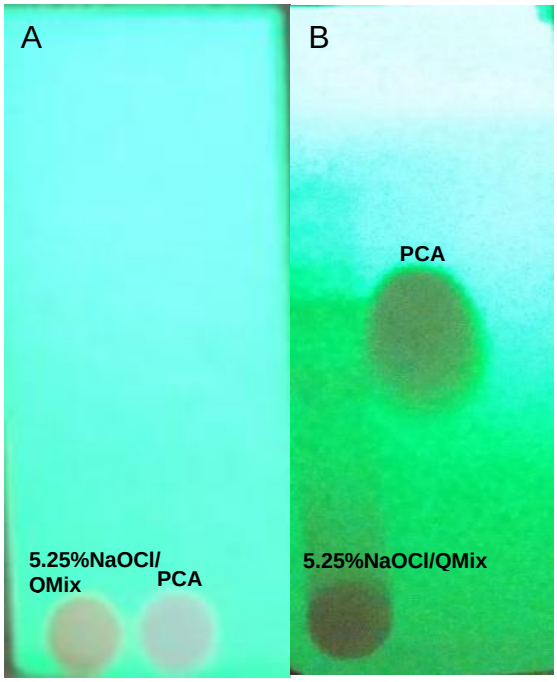

Figure 1. TLC plates under UV light for the 5.25% NaOCl/QMix irrigant association and PCA before (*A*) and after (*B*) development in solvent.
The same picture is obtained for all other investigated irrigants associations.

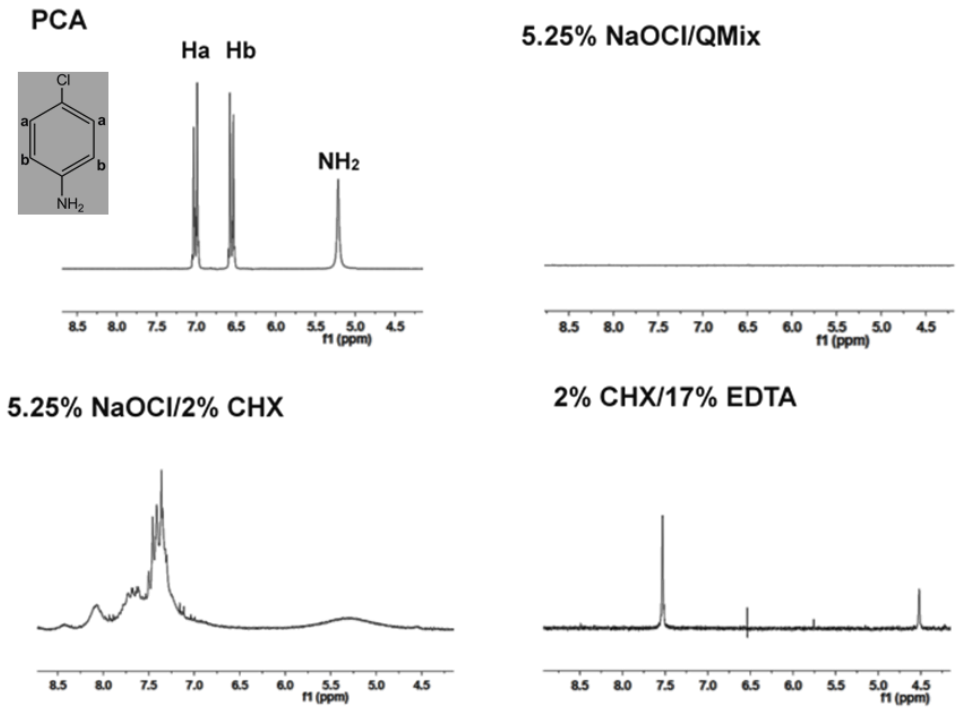

Figure 2. Analysis of investigated irrigants association products by ^{1}H NMR spectroscopy.
All spectra were recorded in DMSO-d_6 at ambient temperature
and compared with that obtained for PCA.

The ^{1}H NMR spectrum for each association product was compared with the spectrum of PCA; see Fig. 2. As can be seen from this figure, two doublet signals at 7.01 and 6.56 ppm with coupling constant J = 8.8 Hz are present in the spectrum of PCA and are assigned to the pairs of aromatic protons H_a and H_b, respectively, while a singlet at 5.22 ppm is due to the NH_2 protons. However, these resonances are not present in the spectra of association products, indicating that no PCA was formed in the investigated reactions. Moreover, there was no appearance of PCA resonances characteristic in association products spectra recorded 72 h after their dissolution.

In the IR spectrum, PCA has two very strong and sharp bands at 3472 and 3382 cm^{-1}, which were assigned to the asymmetric and symmetric stretching vibration of the primary amino moiety (Fig. 3). None of these bands can be detected in the spectrum of the investigated association products, additionally confirming that these products do not contain PCA.

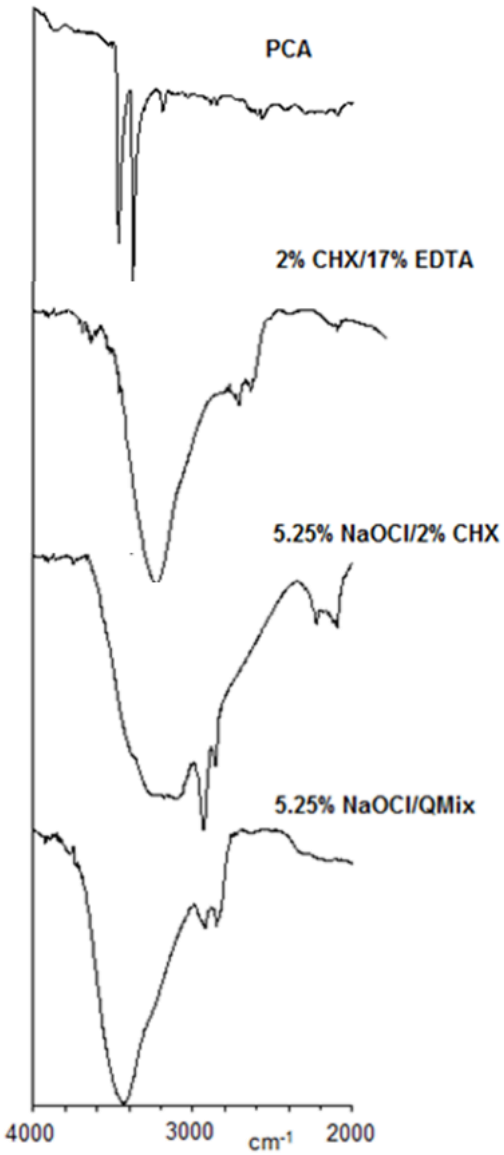

Figure 3. Analysis of the investigated irrigants association products by IR spectroscopy. Their spectra in the region of 4000–2000 cm^{-1} are compared with that for the PCA.

Considering the fact that different irrigants combination during endodontic therapy may enhance their antimicrobial properties (KURUVILLA *et al.,* 1998), possible undesirable chemical interactions among the irrigants have to be investigated (ROSSI-FEDELE *et al.,* 2012). Some composition precipitate findings, resulted from different irrigants combination, showed that there is still a debate whether it contains PCA (TAY *et al.,* 2006; BASRANI *et al.,* 2007; RASIMICK *et al.,* 2008; THOMAS *et al.,* 2010; ARSLAN *et al.,* 2015; ORHAN *et al.,* 2016). Contradictory results can be explained by different detection sensitivities and methods that include handling and dissolution of the precipitate that may affect product recovery. Also, in some studies, failure to detect PCA may be related to the used materials and methods but is not an evidence of its absence (KOLOSOWSKI *et al.,* 2014; ORHAN *et al.,* 2016).

The purpose of this *in vitro* study is to determine whether PCA was formed as a by-product of the reactions between NaOCl and final rinse CHX and QMix solutions, as well as a result of EDTA/CHX combination. This can be important because PCA is considered to be cytotoxic on human cells (WORLD HEALTH ORGANISATION, 2006), carcinogenic (THOMAS *et al.,* 2010) and can lead to neonates methemoglobinemia (MESSMER *et al*., 2015). TLC, ^{1}H NMR and IR methods were chosen for this study due to their great sensitivity in identifying components of a given reaction mixture, as well as determination of mixture purity. Moreover, ^{1}H NMR spectroscopy is one of the principal techniques used to structurally characterize compounds in a nondestructive and noninvasive manner. Previous studies (BASRANI *et al.,* 2007; ORHAN *et al.,* 2016) showed that the use of a destructive method, such as mass spectroscopy, which is based on breaking down of the precipitate, can lead to unreliable results. Therefore, in accordance to previous study by ORHAN *et al.* (2016), noninvasive spectroscopic analyses (^{1}H NMR and IR) and TLC chromatographic method were used for the identification of compounds present in the precipitate.

On the basis of TLC, ^{1}H NMR and IR measurements, we found that a brown precipitate formed in the reaction between 5.25% NaOCl and 2% CHX does not contain PCA. This finding is in accordance with previous studies (KACMAR *et al.,* 1995; SOUZA *et al.,* 2013; Prado *et al.,* 2013), where the absence of PCA in the brown precipitate was confirmed by ^{1}H NMR and ESI-MS analysis. Moreover, NOWICKI *et al.* (2011), reported that NaOCl/CHX association did not produce PCA at any measurable quantity and identified two major CHX breakdown products in NaOCl presence, namely *p*-chlorophenylurea and *p*-chlorophenylguanidyl-1,6-diguanidyl-hexane. Contrary to this, ^{1}H NMR study by BASRANI *et al.* (2007) and ARSLAN *et al.* (2015) showed that NaOCl mixed with CHX resulted in an orange-brown solution coloration and PCA formation. It is considered that the precipitate was formed by the acid-base reaction between CHX, a dicationic acid, that donated protons and NaOCl that accepted protons (BASRANI *et al.,* 2007). This resulted in insoluble precipitate formation that can stain dentin, bond to the walls of the access cavity and root canal (KRISBNAMURTBY *et al.,* 2010). Thus, the precipitate acts as a residual film and chemical smear layer that may compromise intracanal medicaments diffusion, disrupts canal filling adhesion and favours coronal restoration breakdown (BUI *et al.,* 2008; PRADO *et al.,* 2013).

Similarly, in the presence of EDTA, no decomposition of CHX to PCA was observed by TLC and spectroscopic analysis (Fig. 1-3). This is in accordance with the previous HPLC study on the CHX/EDTA association product (RASIMICK *et al.,* 2008), showing that CHX was not decomposed by EDTA and that white product obtained from this reaction represented a salt formed by neutralization of the cationic CHX by the anionic EDTA, according to the equation:

$$3H_2CHX^{2+}(aq) + 2HEDTA^{3-}(aq) \rightleftharpoons (H_2CHX)_3(HEDTA)_2(s)$$

As a result of this interaction, the effect of EDTA on smear layer is reduced, therefore manufacturer's recommendation regarding the use of a root canal saline rinse after NaOCl and before EDTA final rinse should be adopted (RASIMICK *et al.,* 2008).

QMix is a novel final irrigation compound containing CHX, EDTA and the detergent (DAL *et al.* 2011; WANG *et al.* 2012). Our analysis showed that 5.25% NaOCl with QMix combination resulted in orange-brown solution coloration and bubble formation. Analogous to its constituents, QMix does not undergo decomposition to PCA in the presence of 5.25% NaOCl, what is in accordance with the previous finding (ARSLAN *et al.,* 2015). As can be seen from the ^{1}H NMR spectrum of the 5.25% NaOCl/QMix association product, none of the signals in 4.50-8.50 ppm region including those of CHX was detected (Fig. 2). This might have been due to the fact that CHX concentration in QMix is very low, and therefore could not be detected (ARSLAN *et al.,* 2015). Similar results were found by KOLOSOWSKI *et al.* (2014), who showed that the precipitate or PCA were not produced by a mixture of NaOCl and QMix using Time-of-Flight Secondary Ion Mass Spectrometry (TOF-SIMS) analysis. It could be argued that the inability to identify PCA was related to the use of different detection techniques, with a reduced sensitivity to low-molecular-weight products or the use of solvents that may have undermined PCA detection (KOLOSOWSKI *et al.,* 2014; ORHAN *et al.,* 2016).

CONCLUSIONS

The results of our study showed no presence of *p*-chloroaniline as by-product for the none of the investigated irrigants associations, namely 5.25% NaOCl/2% CHX, 2% CHX/17% EDTA and 5.25% NaOCl/QMix. The applied proton NMR and IR spectoscopic techinques and TLC chromatography are shown as very efficient methods of determination whether *p*-chloroaniline is formed in the reactions between presently investigated endodontic irrigants. However, further studies are needed in order to investigate chemical composition of the precipitates formed after previously mentioned associations. Study aimed in this achievement is in progress.

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