Proton Nuclear Magnetic Resonance Spectroscopy Analysis of Mixtures of Chlorhexidine with Different Oxidizing Agents Activated by Photon-Induced Photoacoustic Streaming for Root Canal Irrigation

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Abstract

Objective: The aim of this *in vitro* study was to investigate the possible interactions between photon-induced photoacoustic streaming (PIPS™)-activated oxidizing agents and 2% chlorhexidine digluconate.

Background data: There is no information about the safety of laser-activated oxidizing agents in combination usage with chlorhexidine gluconate.

Materials and methods: Groups were designed as follows G1: 98% para-chloroaniline (PCA); G2: 2% chlorhexidine (CHX); G3: 5.25% sodium hypochlorite (NaOCl) +2% CHX; G4: 5.25% NaOCl (30 sec PIPS activated) +2% CHX; G5: 5.25% NaOCl (60 sec PIPS activated) +2% CHX; G6: 3.5% chlorine dioxide (ClO₂) + 2% CHX; G7: 3.5% (ClO₂) (30 sec PIPS activated) +2% CHX; G8: 3.5% (ClO₂) (60 sec PIPS activated) +2% CHX. The laser-irrigation protocol was performed with an erbium:yttrium–aluminum–garnet laser with a wavelength of 2940 nm equipped with a 140 mm long endodontic fiber tip (PIPS) using 10 mJ at 15 Hz (0.15 W), per pulse operating outputs. Groups were analyzed with proton nuclear magnetic resonance spectroscopy, using PCA as an internal standard.

Results: No free PCA was formed in any groups of mixtures or after PIPS activation.

Conclusions: Mixing of 3.5% ClO₂ and 2% CHX does not form bulky precipitates, unlike the mixture NaOCl + CHX. PIPS activation does not cause changes in reactions of oxidizing agents.

Keywords: chlorhexidine, chlorine dioxide, NMR spectroscopy, photon-induced photoacoustic streaming, sodium hypochlorite

Introduction

THE PURPOSE OF endodontic treatment is to remove pulp tissue and microorganisms from root canals. Because of the complicated anatomy of root canals, uncleaned canal space can remain after root canal instrumentation.¹ Thus, root canal irrigation is a crucial step for root canal disinfection and debris removal.² Sodium hypochlorite (NaOCl) is one of the most widely used irrigation solutions in root canal treatment because of its antibacterial effect and organic tissue dissolution capacity. However, the efficiency of NaOCl is directly related to its concentration. The NaOCl should be used at low concentration because it can be toxic on periapical tissues at high concentrations.^{3,4} However, there is decreased

efficiency of NaOCl at lower concentrations. The efficiency may be regained with strategies such as agitation/activation methods or combination with additional agents. In addition, studies are underway to discover alternative oxidizing agents to hypochlorite to eliminate its disadvantages.

Chlorine dioxide (ClO₂) is a commonly used biocompatible disinfectant, bleaching solution, and oxidizing agent. It is used in the food industry, veterinary medicine, water treatment, surface disinfection, and mouth care products.^{5–7} ClO₂ at low concentrations is able to dissolve organic tissues without causing toxic or allergenic reactions.^{8,9} A recent study has reported that ClO₂ may be an alternative root canal irrigation agent in root canal treatment instead of NaOCl because of its antimicrobial activity, biocompatibility, and

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organic dissolution capacity.^{8–10} Throughout its oxidation process, toxic byproducts are not formed.¹⁰

Laser-activated irrigation (LAI) has been introduced for activating irrigation solutions through the transfer of pulsed energy.^{11,12} In principle, photon-induced photoacoustic streaming (PIPSTM) is an LAI technique utilizing minimal ablative energy levels. PIPS can improve the efficiency of irrigation solutions at lower concentrations.¹³ The PIPS technique utilizes an erbium:yttrium–aluminum–garnet (Er:YAG) laser source, including a 3 mm polyamide sheath and a tapered tip, designed for access and cavity penetration. The Er:YAG laser source uses subablative parameters. Thus, it is able to activate the total amount of irrigation solution within the root canal. PIPS generates strong photoacoustic shock waves into the irrigation solution.¹³ Therefore, the PIPS technique has been reported to increase the cleaning efficiency of the root canal system better than conventional needle irrigation.^{14,15}

Chlorhexidine (CHX) is a broad-spectrum antimicrobial agent. Low concentrations are commonly used as oral antiseptics and root canal disinfectant.¹⁶ CHX is the preferred agent for use during the final irrigation because of its residual antimicrobial effects on dentine walls.^{17,18} However, CHX should not be introduced after the NaOCl irrigation without intermittent flushing/dilution in the root canal. When CHX and NaOCl are mixed without dilution, a brown-colored precipitate is formed. This precipitate is hard to eliminate from the root canal walls.¹⁹ There are conflicting results about the molecular structure of the brown precipitate.^{20–22} Para-chloroaniline (PCA) or 4-chloroaniline is a degradation product of CHX molecular structure.^{20,21} However, Orhan et al.²² showed that free PCA did not form after the interaction between NaOCl and CHX. PCA is a toxic and carcinogenic molecule, but fortunately, CHX is not degraded to free PCA after interacting with NaOCl.^{20,22} However, the effects of PIPS-assisted LAI on irrigation agents are still unknown.

The aim of this study was to analyze the PIPS-activated oxidizing agents and their interactions with CHX. The first null hypothesis was that the oxidation agents and their CHX mixtures did not contain free PCA. The second null hypothesis was that PIPS activation did not affect the interactions of irrigation solutions.

Materials and Methods

Preparation of solutions

The following stock solutions manufactured for dental purposes were used: 5.25% fresh NaOCl (aq) (Promida Co., Eskişehir, Turkey) and 2% CHX (aq) (ProChex; Promida Co.). Commercially accessible 3.5% fresh ClO₂ (aq) (CemmoX, Denizli, Turkey) and 98% PCA (4-chloroaniline; Sigma-Aldrich, St. Louis, MO) in powder form were also used for experiments.

Mixtures were created by introducing 1 mL 2% CHX solution to 1 mL chlorinated solution per group in Eppendorf tubes. The mixture formed by the addition of two solutions was adjusted to 2 mL. Solutions were mixed in Eppendorf tubes at 25°C. Additional solutions were delivered using an automatic pipette (Maxipettor Eppendorf; Thomas Scientific, Swedesboro, NJ) and mixing by using a capillary laboratory pipette for 60 sec.

PIPS protocol

The previously described laser-irrigation protocol was performed with an Er:YAG laser with a wavelength of 2940 nm (LightWalker; Fotona, Ljubljana, Slovenia) equipped with a 140 mm long endodontic fiber tip of PIPS using 10 mJ at 15 Hz (0.15 W), per pulse operating outputs.¹³ The fiber tip was placed 3 mm deep into the solutions to simulate clinical usage. Total activation time was either 30 or 60 sec, as suggested by various authors who use PIPS.^{23,24} The PIPS was operated in 1 mL of solution in accordance with suggestions by authors who use LAI.

pH measurement

The pH value and temperature of each tested solution was measured using a pH meter (WTW inoLab 7110; Weilheim, Germany) before PIPS activation, after 30 and 60 sec of PIPS activation, and after mixing.

Extraction of precipitates

A previous extraction and solvation method was used in this study using analytical grade ethyl acetate.^{22,25} Precipitates were extracted three times in 15 mL of ethyl acetate and were added into an organic solvent. The organic layer of the precipitates was detached and dehydrated with Na₂SO₄. The solvent was removed, and the solid part of the precipitates was then obtained under reduced pressure.

Proton nuclear magnetic resonance (¹H NMR) spectroscopy

Nuclear magnetic resonance (NMR) spectra were obtained using a JEOL RESONANCE Spectrometer (ECZ 500R; JEOL Co., Ltd., Tokyo, Japan) utilizing a 5 mm BBO probe at 298 K and 11.75 T. The operating frequency was 500.13 MHz for a ¹H nucleus. Each spectrum was recorded in deuterated dimethyl sulfoxide (d₆-DMSO) separately using C₄H₁₂Si as an external standard in a ¹H NMR spectroscopy unit.²²

Results

The color of each group was as follows: 2% CHX and 5.25% NaOCl were colorless; 3.5% ClO₂ was yellowish,

FIG. 1. Appearances of the solutions prepared (left to right): 5.25% NaOCl; 3.5% ClO₂; 2% CHX; 5.25% NaOCl mixed with 2% CHX; 5.25% NaOCl activated by 30 sec PIPS, mixed with 2% CHX; 5.25% NaOCl activated by 60 sec PIPS, mixed with 2% CHX; 3.5% ClO₂ mixed with 2% CHX; 3.5% ClO₂ activated by 30 sec PIPS, mixed with 2% CHX; 3.5% ClO₂ activated by 60 sec PIPS, mixed with 2% CHX. CHX, chlorhexidine; ClO₂, chlorine dioxide; NaOCl, sodium hypochlorite; PIPSTM, photon-induced photoacoustic streaming.

TABLE 1. EXPERIMENTAL GROUPS

No.	Groups	Precipitate	Color
1	98% PCA (solid)	N/A	White
2	2% CHX (aqueous solution)	N/A	Noncolored
3	5.25% NaOCl +2% CHX	Yes	Brownish
4	5.25% NaOCl +30 sec PIPS +2% CHX	Yes	Brownish
5	5.25% NaOCl +60 sec PIPS +2% CHX	Yes	Brownish
6	3.5% ClO ₂ + 2% CHX	No	Straw-colored or yellowish
7	3.5% ClO ₂ + 30 sec PIPS +2% CHX	No	Straw-colored or yellowish
8	3.5% ClO ₂ + 60 sec PIPS +2% CHX	No	Straw-colored or yellowish

CHX, chlorhexidine; ClO₂, chlorine dioxide; NaOCl, sodium hypochlorite; PCA, para-chloroaniline or 4-chloroaniline; PIPS™, photon-induced photoacoustic streaming with erbium:yttrium–aluminum–garnet activation.

3.5% ClO₂ + 2% CHX, 3.5% ClO₂ + 30 sec PIPS +2% CHX, and 3.5% ClO₂ + 60 sec PIPS +2% CHX were straw colored; an 5.25% NaOCl +2% CHX, 5.25% NaOCl +30 sec PIPS +2% CHX, and 5.25% NaOCl +60 sec PIPS +2% CHX were brownish (Fig. 1 and Table 1).

The pH values of the single solutions at room temperature are given in Table 2. The pH values of the mixtures at room temperature are given in Table 3. After 30 and 60 sec of PIPS activation, the temperatures and pH values of the solutions increased. Although CHX was not activated by PIPS during the experiment, pH and temperature changes were also examined separately.

¹HNMR spectral data are represented in Fig. 2. In 98% PCA, the spectrum revealed two doublets at 7.00 and 6.55 ppm for an aromatic ring and one singlet signal for an amino (-NH₂) group at 5.23 ppm.^{22,25} The datasets (raw spectral data) are also available in the Supplementary Data.

The d₆-DMSO peaks were observed as a singlet at 2.5 ppm, and water peaks were observed at 3.37 ppm in all spectra. In NaOCl mixtures, both the aromatic and the aliphatic region of CHX changed. The shifts in the aromatic region were recorded between 7.30 and 7.80 ppm. The doublet observed in the aliphatic region of the CHX spectrum between 0.9 and 1.1 ppm and a broad peak at 2.95 ppm were lost after the NaOCl mixture was added. In ClO₂ mixtures, the aliphatic region of CHX was less affected, and the aromatic region changed only minimally. There was a reaction shown only by peaks between 6 and 8 ppm, and the peaks between 9 and 10 ppm disappeared in the aromatic region.

¹HNMR spectral data revealed that no free PCA existed in any solutions, mixtures, or precipitates. The PIPS activation did not cause changes in the structure of precipitates or mixtures.

TABLE 2. pH AND TEMPERATURES OF SOLUTIONS

Groups	ClO ₂	NaOCl	CHX
Initial			
pH	4.74	9.26	5.96
°C	26.2	26.2	26.2
30 sec PIPS			
pH	4.75	9.27	6.00
°C	26.8	26.8	26.8
60 sec PIPS			
pH	4.75	9.27	6.01
°C	27.8	27.7	27.7

Discussion

NMR spectroscopy can examine molecules without integrating their molecular structures. NMR spectral analysis allows comparing peak intensity and location for fine-tuned concentration values of any experimental spectrum. When signals are arranged at the same pH or field strength, then the experimental material is verified to exist in that compound. Therefore, the mixtures and standard molecules were analyzed with their HNMR spectral data (singlet, doublet, etc.) in this study and compared. All spectral data were recorded under the same magnetic field strength at 500.13 MHz and all analyses were performed individually. No databases or libraries containing spectral data were used.

The ¹HNMR spectra of pure PCA are fundamental to visualizing an amino (-NH₂) signal at 5–5.5 ppm.^{22,25–27} Therefore, Thomas and Sem used pure PCA as the “internal standard” or “control” in their ¹HNMR spectroscopy study.²⁶ Spectral data (peaks of the spectrum pattern as a singlet, doublet, etc.) can only be compared with an internal standard or spectral databases. In previous studies, Orhan et al.,²² Irmak et al.,²⁵ and Nocca et al.²⁸ used pure PCA as an internal standard in ¹HNMR spectroscopy analysis of the same kind of precipitate. In other literature, the spectral analyses of the irrigation agents and their interactions were evaluated using only a single sample per experimental group. There have not been any exceptions to this sample quantity in the literature.^{22,25,26} Further comparisons with other interaction scenarios could be considered in future designs.

A characteristic of the aromatic subspectrum of 98% PCA is that it has a primary amino (-NH₂) singlet at 5.0–5.5 ppm;^{22,25} thus, existence of an -NH₂ singlet indicates free PCA in the experimental material. The mixture groups and 2% CHX revealed dissimilar aromatic doublet peaks and a singlet of 98% PCA. Regardless of the oxidizing agent, we did not detect free PCA in 5.25% NaOCl, CHX mixtures, or 2% CHX. Our results are in agreement with previous reports.^{22,25,26} Therefore, the first null hypothesis is accepted.

The specific amino signals of the ¹HNMR spectrum for 98% PCA were compared with those from the mixture of

TABLE 3. pH AND TEMPERATURES OF MIXTURES

Groups	pH	°C
ClO ₂ + CHX	4.78	27.8
NaOCl + CHX	9.75	27.7

FIG. 2. ^1H NMR experiments showed no free PCA was found in the degradation of CHX: (a) Reference spectrum of PCA in $\text{d}_6\text{-DMSO}$. (b) Reference spectrum of 2 mL 2% CHX in $\text{d}_6\text{-DMSO}$. (c) ^1H NMR spectrum acquired after addition of 1 mL 2% CHX (aq) to 1 mL 5.25% NaOCl (aq). (d) ^1H NMR spectrum acquired after addition of 1 mL 2% CHX (aq) to activated 1 mL 5.25% NaOCl (aq) by 30 sec PIPS. (e) ^1H NMR spectrum acquired after addition of 1 mL 2% CHX (aq) to activated 1 mL 5.25% NaOCl (aq) by 60 sec PIPS. (f) ^1H NMR spectrum acquired after addition of 1 mL 2% CHX (aq) to 1 mL 3.5% ClO_2 (aq). (g) ^1H NMR spectrum acquired after addition of 1 mL 2% CHX (aq) to activated 1 mL 3.5% ClO_2 (aq) by 30 sec PIPS. (h) ^1H NMR spectrum acquired after addition of 1 mL 2% CHX (aq) to activated 1 mL 3.5% ClO_2 (aq) by 60 sec PIPS. $\text{d}_6\text{-DMSO}$, deuterated dimethyl sulfoxide; PCA, para-chloroaniline.

2% CHX and 3.5% ClO_2 for the first time in this study. When 2% CHX was added to 3.5% ClO_2 , no precipitation occurred. However, a weak nonacid–base reaction was observed as a result of the mixture; the color of the 3.5% ClO_2 turned from a yellowish color to a cloudy straw color. Previous studies have reported the characteristics of ClO_2 . It is antibacterial, it dissolves organic tissue, and it is nontoxic.^{8–10} However, there is limited information about the reactions of ClO_2 . Herczegh et al.²⁹ reported the results of an *in vitro* study using an high performance liquid chromatography–mass spectrometry (HPLC/MS) method, which showed that free PCA did not appear after mixing of 1% CHX and 0.12% hyper-pure ClO_2 . That supports our findings on free PCA. Although NaOCl and ClO_2 are both oxidizing agents, they could have different reactions with CHX.³⁰ In agreement with that previous report, precipitation-like reactions did not occur in our study. A brown-colored precipitate could have a negative influence on the penetration of root canal sealer on dentin tubules and could cause discoloration. It could also be difficult to remove.¹⁹ Thus, the precipitation could negatively affect the

root canal filling and postendodontic restoration quality.¹⁹ The baseline mixing procedures were tested in our study, and free PCA was analyzed using HNMR spectroscopy. Further studies are needed to understand the clinical effects of ClO_2 and its combinations with other irrigation agents on root canal walls. A previous study that compared 2.5% NaOCl and 13.8% ClO_2 found that 2.5% NaOCl caused roughness of the root canal wall.³⁰

In this study, it was observed that pH values of oxidizing agents increased by 1% with PIPS activation. The effect of pH change on chlorine-containing compounds has been previously investigated, and it has been reported that this affects their organic-tissue-dissolving and antibacterial properties.³¹ However, because an increase of 1% does not significantly change the alkaline or acid properties of the solution, it can be concluded that PIPS activation has no effect on pH within the parameters of our study. However, further studies are needed on this subject.

This study found increased temperature and pH of CHX after PIPS activation. Although the pH of CHX increased

with PIPS activation in this study, it is known that the optimal antibacterial effect of CHX occurs between pH 5.5 and 7.³² Mixtures of PIPS-activated CHX were not examined in this study, but the effects of PIPS on all solutions used in the experiment were investigated separately. Basrani et al. investigated the effect of temperature changes on CHX and reported that CHX turned white when heated to 37°C, but a yellowish color was observed at 45°C.³³ However, further studies on the effects of pH and temperatures are needed.

Different agitation techniques are used to increase the effectiveness of endodontic irrigation solutions.¹² PIPS activation has been reported to be more efficient than the other activation methods.¹¹ Gunesser et al. have reported that PIPS activation of 5.25% NaOCl increases the efficiency of organic tissue dissolution more than a technique using a sonic activation system and an Er:YAG laser using an endodontic fiber tip activation.³⁴ Similarly, Peters et al. have reported PIPS activation of 6% NaOCl eliminated more biofilm than ultrasonic activation and conventional irrigation techniques.¹² Golob et al. have reported that PIPS activation of 5% NaOCl is an effective method for elimination of *Enterococcus faecalis*.³⁵ In the PIPS technique, the use of subablative energy (20 mJ) produces a high peak power of 400 W, causing an explosion–implosion phenomenon within the irrigation solution. The primary thermal effect of laser energy absorbed in irrigation solution leads to superheating of the irrigant up to the boiling point and formation of vapor bubbles.³⁵ This phenomenon explains the temperature changes in this study. ClO₂ is a highly unstable molecule. Therefore, ClO₂ reactions were tested by using PIPS activation as an efficient agitation method. Although ClO₂ temperature increases, the PIPS activation did not cause any change in the structure of the precipitates and mixtures in this study. Thus, the second null hypothesis is accepted. The mixture of PIPS-activated 3.5% ClO₂ and 2% CHX was analyzed. In this experiment, 3.5% ClO₂ mixtures did not form brown precipitates; instead, the color of the mixtures was observed to turn into cloudy light yellow.

Conclusions

Within the limitations of this *in vitro* study the following conclusions can be drawn:

- Mixing of 3.5% ClO₂ and 2% CHX does not form any precipitate.
- The precipitate formed after 5.25% NaOCl and 2% CHX 11 mixed does not yield free PCA.
- PIPS activation does not cause changes in reactions of oxidizing agents.

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Supplementary Material

Supplementary Data

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