Decoding the Perplexing Mystery of Para-Chloroaniline Formation: A Systematic Review

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Objective: The objective of this article was to understand and decode the mystery of the formation of para-chloroaniline (PCA). The ingredient of the brown precipitate after mixing sodium hypochlorite (NaOCl) and chlorhexidine gluconate (CHX) is still in debate. Materials and Methods: Various studies adopt a different methodology to substantiate that it may contain PCA, which is a carcinogenic agent. The purpose of this systematic review is to evaluate the relationship between PCA and brown precipitate. Two reviewers independently conducted a comprehensive literature search. The MEDLINE, Embase, Cochrane, and PubMed databases were searched. In addition, the bibliographies were manually searched. There was no disagreement between the two reviewers. This review was reported and conducted in step with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines. Results: Of 233 articles, only 13 articles met the inclusion criteria. Available scientific evidence was more supportive that the brown precipitate form after mixing NaOCl and CHX may form para-chloroamide moiety rather than free PCA, and PCA may be the by-product of CHX degradation. Conclusion: On the basis of the current evidence and data extracted from the various databases, it can be concluded that the mixture of sodium hypochlorite and chlorhexidine does not form PCA, and PCA may be the by-product of high concentrated chlorhexidine. Further studies are required to substantiate the evidence.

Keywords: Chlorhexidine, para-chloroaniline, sodium hypochlorite, spectroscopy

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INTRODUCTION

Persistent infection due to remnant infected dentinal debris bacteria is the cause of root canal failure.¹ Therefore, to obtain a sterilized canal, irrigation plays a crucial role.² The most common irrigant with antimicrobial property is sodium hypochlorite (NaOCl), but the increased concentration of NaOCl can be potentially toxic to the periapical tissue.³,⁴ Chlorhexidine gluconate (CHX) was introduced as an alternative against NaOCl with the good antimicrobial property but a lack of tissue dissolution capacity.⁵-⁷ The introduction of chlorhexidine in the presence of NaOCl into the canal leads to the formation of brown precipitate due to acid–base reaction that extends up to 139–684 µm, which deteriorate the sealing of the root canal.⁸ Some of the previously published studies claim the presence of the para-chloroaniline (PCA) in the brown precipitate,⁹-¹² but another study denies the concept of the formation of PCA.¹³,¹⁴

Previous study was unable to substantiate the formation of PCA. It is important to understand
that mixing NaOCl and CHX forms PCA or not because PCA known to be carcinogenic and may cause methemoglobinemia.\textsuperscript{[15-19]}

To date, the formation of PCA due to NaOCl and CHX remains vague. Therefore, the purpose of this systematic review was to evaluate whether PCA is formed through the reaction of mixing NaOCl and CHX.

\textbf{MATERIALS AND METHODS}

The protocol for this systematic review was developed following established guidelines.\textsuperscript{[20]} The protocol was prepared and matched the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines for systematic reviews. The focused question was adjusted according to the PICO (Population, Intervention, Comparator, Outcome) criteria for comparing laboratory studies as follows: Population (specimens or extracted teeth); Intervention and Comparison (interaction between NaOCl and CHX); Outcome (PCA formation).\textsuperscript{[21]}

\textbf{SEARCH STRATEGY}

A literature search was performed using the U.S. National Library of Medicine (MEDLINE), Embase, Cochrane, and PubMed databases. Articles were included up to and including August 29, 2019.

In addition, bibliography of articles and textbooks were manually searched. Two reviewers (M.S. and B.A.) independently searched the article and abstract based on inclusion and exclusion criteria. All potential studies were analyzed and assessed completely. Only studies published in English were included.

\textbf{INCLUSION CRITERIA}

\textit{In vitro} studies were considered and studies evaluating PCA formation were included.

\textbf{EXCLUSION CRITERIA}

Case series, cell culture laboratory studies, or animal studies were excluded.

\textbf{RESULTS}

Flowchart of this systematic review based on PRISMA guidelines is presented in Figure 1. Of 233 studies, 13 met the inclusion criteria.

\textbf{STUDY SELECTION}

After the database screening and the removal of duplicates, 233 studies were identified. After screening the titles, 22 were left. It was further reduced to 13 after the examination of abstracts and full texts.

\textbf{CHARACTERISTICS OF INCLUDED ARTICLES}

This review includes studies published in English. The characteristics of 13 included studies are listed in Tables 1 and 2. Ten articles focused on the combination of NaOCl and CHX to analyze the formation of PCA, and the remaining three articles analyzed degradation and stability of CHX at various concentrations.

The various analytical methodologies used to detect PCA formation are listed in Table 3.

\textbf{DISCUSSION}

This systematic review focused on any association between the identification of PCA and the analytical methods used for identification in various literature.

\textbf{GAS CHROMATOGRAPHY/MASS SPECTROMETRY}

Three articles were identified to address a relationship between PCA formation and gas chromatography–mass spectrometry.\textsuperscript{[10,13,14,22-24]} Two articles reported that the combination of NaOCl and CHX leads to the formation of PCA.\textsuperscript{[10,13]} Previous studies analyzed that CHX degradation at various concentrations liberates PCA,\textsuperscript{[22-24]} whereas Orhan \textit{et al.}\textsuperscript{[14]} reported that for the detection of PCA, mass spectrometry is not an appropriate method.

\textbf{1H NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY}

Five articles were identified, which assessed the PCA formation.\textsuperscript{[12,14,25-27]} Even though 1H nuclear magnetic resonance (NMR) spectroscopy is a nondestructive method for detecting PCA, results of various studies vary. Three studies clearly mentioned the absence of free PCA in the NaOCl and CHX reaction.\textsuperscript{[14,25,27]} On the contrary, two studies supported the formation of PCA.\textsuperscript{[12,20]} The spectrum interpretation performed between the range of 6.5–8.5 ppm followed by concluded that 7.0–8.0 ppm represent PCA but spectra result of the amino signal was included in the discussion. We contemplate that the inaccuracy of the results could be due to misinterpretation of para-chloro amide moiety of CHX or any derivative of CHX in the brown precipitate.\textsuperscript{[14]} Orhan \textit{et al.} single out the methodological error of the study by Arslan \textit{et al.}\textsuperscript{[20]} and bring down the curtain with the statement that the 1H NMR analysis was misunderstood and the result was misinterpreted.\textsuperscript{[29]}

\textbf{HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND THIN LAYER CHROMATOGRAPHY}

Two articles assessed the PCA formation in NaOCl and CHX reaction.\textsuperscript{[12,14]} Even tough high-performance liquid chromatography is a nondestructive method to analyze the PCA formation, the results of both studies
are completely different. Siddique et al.\textsuperscript{[12]} supported the formation of free PCA but Orhan et al.\textsuperscript{[14]} opposed it. However, Siddique et al.\textsuperscript{[12]} were unable to mention para-chloro amide moiety of CHX. Therefore, their verification to justify free PCA formation may be inaccurate.

**Time-of-flight secondary ion mass spectrometry**

Two articles supported the PCA formation using time-of-flight secondary ion mass spectrometry (TOF-SIMS).\textsuperscript{[9,11]} The presence of PCA can be interpreted using TOF-SIMS but unable to differentiate between free PCA and amide moiety. TOF-SIMS showed the presence of PCA in the CHX group and NaOCl/CHX group. Hence TOF-SIMS cannot be a reliable method for detecting PCA.

**Infrared spectroscopy**

Two published studies examined PCA formation in the NaOCl/CHX mixture, and both studies denied PCA formation in the brown precipitate.\textsuperscript{[14,27]}

**Column chromatography, electron spray ionization mass spectrometry, and ultraviolet**

Only one study used column chromatography, electron spray ionization mass spectrometry, and ultraviolet to analyze the presence of PCA in the mixture.\textsuperscript{[13]} However, this study discussed the value range for PCA but not about amide moiety; thus, the result of this study cannot be a cutoff proof for finding PCA in the mixture.

**Stereomicroscopy**

Krishnamurthy and Sudhakaran.\textsuperscript{[28]} detected to confirm the presence of PCA using stereomicroscopy and NMR and concluded that intermediate flushing with saline can prevent the formation of PCA.

**X-ray photon spectroscopy**

Basrani et al.\textsuperscript{[9]} confirmed the presence of PCA using X-ray photon spectroscopy and TOF-SIMS.

Neither Krishnamurthy and Sudhakaran\textsuperscript{[28]} nor Basrani BR et al.\textsuperscript{[9]} discussed para-chloro amide

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**Figure 1: Flow diagram based on PRISMA guideline**

[Diagram showing the flow of studies through PubMed, Medline and Embase, and Cochrane, with details on the number of studies at each stage and the final selection criteria.]

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Khatib, et al.: Decoding the perplexing mystery of para-chloroaniline
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moiety of CHX or CHX derivative. Zong and Kirsch [30] tested the pH-dependant chlorhexidine instability and concluded that chlorhexidine degradation into PCA is more in an acidic medium as compared to the alkali medium, which could be the cutoff proof that NaOCl is not responsible for PCA formation due to its alkali nature.

NaOCl and CHX lead to an acid–base reaction that forms brown precipitate, which may contain para-chloroamide moiety indeed it disturbs the sealer penetration and may affect periapical seal, [24] or it is due to NaOCl that cause chlorination of the guanidino nitrogen of CHX. [32] But to date no conclusive methodology is available that clearly identify the by-product of NaOCl.

### Table 1: Studies focused on NaOCl and CHX mixture to analyze PCA formation

| Study ID         | Study parameters | Analysis method                        | Outcome                                      |
|------------------|------------------|----------------------------------------|----------------------------------------------|
| Basrani et al.  | In vitro         | XPS and TOF-SIMS                        | PCA or isomers found in the precipitate      |
| Thomas and Sem   | In vitro         | GC-MS                                  | PCA was found in the precipitate             |
| Mortenson et al. | In vitro         | TOF-SIMS                                | Presence of PCA in dentinal tubule           |
| Ahmad et al.     | In vitro         | 1H NMR spectroscopy                    | Presence of PCA in NaOCl and CHX mixture.    |
| Orhan et al.     | In vitro         | HPLC, proton NMR, gas chromatography,   | No PCA was found in the precipitate          |
| Irmak et al.     | In vitro         | 1H proton NMR and IR spectroscopy      | No PCA was found in the precipitate          |
| Siddique et al.  | In vitro         | TLC, HPLC, CC, ESIMS, UV, and NMR (1H  | PCA was found in the precipitate             |
|                  |                  | NMR and C-13 NMR)                      |                                              |

**Table 1 Notes:**

- GC-MS = gas chromatography–mass spectrometry, HPLC = high-performance liquid chromatography, IR = infrared, TLC = thin-layer chromatography, TOF-SIMS = time-of-flight secondary ion mass spectrometry, UV = ultraviolet, XPS = X-ray photon spectroscopy

### Table 2: Studies focused on CHX concentration to analyze PCA formation

| Study ID         | Study parameters | Analysis method                        | Outcome                                      |
|------------------|------------------|----------------------------------------|----------------------------------------------|
| Barbin et al.    | In vitro         | Mass spectrometry, HPLC                | ROS liberation was seen with the combination of CHX and Ca(OH)₂ |
| Barbin et al.    | In vitro         | GC-MS                                  | PCA and ROS were the by-products of the 2% CHX aqueous solution |
| Câmara De Bem et al. | In vitro     | GC-MS                                  | 2% CHX gel and solution may degrade into PCA, oCA, mCA, ROS, and organochlorines (ortho-chlorophenyl isocyanate and 2-amino-5-chlorobenzonitrile) regardless of storage conditions or time |

**Table 2 Notes:**

- GC-MS = gas chromatography–mass spectrometry, HPLC = high-performance liquid chromatography, mCA = meta-chloroaniline, oCA = ortho-chloroaniline, ROS = reactive oxygen species
and CHX mixture. Many studies have been performed to solve the mystery of the brown precipitate; some studies supported and some opposed the PCA formation. It may be due to differences between the techniques; however, based on available data it is clear that the brown precipitate might contain aromatic ring, which might be the substitute of PCA.[25]

There is a concern that the brown precipitate, which is PCA according to some authors, is carcinogenic. Patil.[31] investigated the mutagenic potential of the brown precipitate by Ames test and concluded that the precipitate is free from carcinogenic elements.

Thus, from this systematic review, it can be concluded that the PCA may be the degradation product of concentrated CHX and not the NaOCl/CHX mixture. In future, well-designed research methodologies are needed to address this question.

**CONCLUSION**

On the basis of the provided evidence, we can conclude that NaOCl and CHX mixture does not contain free PCA.

We should understand that leaving the brown precipitate in the canal is terrible, so intermittent flushing should be performed to avoid precipitate formation.

Well-designed studies may able to provide cutoff proof of the absence of free PCA in NaOCl and CHX mixture.

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**CONFLICTS OF INTEREST**

There are no conflicts of interest.

**AUTHOR CONTRIBUTIONS**

Mohd. Sibghatullah Khatib: Concepts, Design, Literature search. Bilal Ameer: Manuscript preparation. Nikita Ajit Mannur: Literature search. Amith Madi Ramalingaiahsetty: Data acquisition. Sayed Mateen Peerzade: Literature search. Amrut Bambawale: Manuscript review.

**ETHICAL POLICY AND INSTITUTIONAL REVIEW BOARD STATEMENT**

To the best of the author(s) knowledge, this study has been conducted in full accordance with the World Medical Association Declaration of Helsinki.

**PATIENT DECLARATION OF CONSENT**

For the further development of medical treatment in dentistry, the publishing of clinical pictures and treatment methods is indispensable. This is why I explicitly agree that all information collected during the course of the treatment, including picture, sound and video material – even if my person / child is recognizable – may be published for scientific as well as for educational purposes in the publishing group of JISPCD. The material may be linked with information about the disease pattern and the treatment methods, etc.

**DATA AVAILABILITY STATEMENT**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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| Analytical method | Articles identified |
|-------------------|---------------------|
| GC-MS             | Barbin *et al.*[22] |
|                   | Basrani *et al.*[10]|
|                   | Mortenson *et al.*[13]|
|                   | Barbin *et al.*[23]|
|                   | Câmara De Bem *et al.*[24]|
|                   | Orhan *et al.*[14]|
| 1H NMR spectroscopy | Thomas and Sem[25] |
|                   | Arslan *et al.*[26]|
|                   | Orhan *et al.*[14]|
|                   | Irmak *et al.*[27]|
|                   | Siddique *et al.*[12]|
| HPLC and TLC      | Orhan *et al.*[14]|
|                   | Siddique *et al.*[12]|
| TOF-SIMS           | Basrani *et al.*[9]|
|                   | Kolosowski *et al.*[11]|
| Infrared spectroscopy | Orhan *et al.*[14]|
|                   | Irmak *et al.*[27]|
| CC                 | Siddique *et al.*[12]|
| ESI-MS             | CC = column chromatography, ESI-MS = electron spray ionization mass spectrometry, GC-MS = gas chromatography–mass spectrometry, HPLC = high-performance liquid chromatography, TLC = thin-layer chromatography, TOF-SIMS = time-of-flight secondary ion mass spectrometry, XPS = X-ray photon spectroscopy

XPS = Basrani *et al.*[9]
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