INTERNATIONAL JOURNAL OF APPLIED PHARMACEUTICS

THE ANTIBACTERIAL POTENTIAL OF N-ACETYLCYSTEINE AS AN ENDODONTIC IRRIGANT ON THE CLINICAL ISOLATES OF THE *ENTEROCOCCUS FAECALIS* BIOFILM

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ABSTRACT

Objective: The aim of this study was to evaluate the antibacterial potential of NAC as an endodontic irrigant on the clinical isolates of the *Enterococcus faecalis* biofilm.

Methods: NAC with a pH of 2.5 and 11 (NAC pH 2.5 and NAC pH 11, respectively) were exposed to clinical isolates of *E. faecalis* biofilms for 1 min. The NAC samples were compared to 2% chlorhexidine (CHX), which is commonly used as an irrigant in persistent infections. The antibacterial potential of these irrigants was evaluated by comparing the bacterial count of the *E. faecalis* colonies after they were exposed to the irrigants.

Results: The NAC pH 2.5 test group showed a reduction in the *E. faecalis* colonies, but this reduction was not statistically significant when compared to the 2% CHX group results. The NAC pH 11 test group showed the greatest reduction in bacterial colonies, and this reduction was statistically significant when compared to the NAC pH 2.5 and 2% CHX groups' results.

Conclusion: NAC pH 11 has antibacterial potential on the clinical isolates of *E. faecalis* biofilms.

Keywords: *Enterococcus faecalis*, Biofilm, Irrigation, N-acetylcystein.

INTRODUCTION

Enterococcus faecalis is commonly involved in secondary and persistent endodontic infections. This bacterium has an up to 90% prevalence in cases of root canal-treated teeth. Root canal-treated teeth are about nine times more likely to harbor *E. faecalis* than cases with primary infections [1]. *E. faecalis* bacteria have various genetic forms. They are able to adapt to minimum nutrition conditions and accumulate and produce biofilms. In the root canal system, the essential nutrients and substances needed by the bacteria for metabolism are passed passively through water channels or by electrostatic interactions, trapping these substances inside the biofilms. It is this biofilm that helps other bacteria in the root canal become more resistant to phagocytosis, antibodies, and antibacterial agents [2].

In conditions with minimum oxygen concentration, the bacteria located in the deepest part of the biofilm will experience phenotypic changes associated with their growth rate and gene transcription, which support the survival and virulent characteristics of the bacteria [3]. Studies show longer survivability of some clinical isolates of *E. faecalis* in pH 12.5 environments compared to *E. faecalis* ATCC 29212 [4]. In addition, the production of hemolysin in the *E. faecalis* clinical isolates was higher than that of *E. faecalis* ATCC 29212 [5]. These findings support the presence of characteristic differences between bacteria derived from clinical isolates and ATCC bacteria and illustrate why it is more difficult to eliminate the clinical isolates of *E. faecalis* than *E. faecalis* ATCC.

Antibacterial agents in the cleaning and shaping procedures of root canal treatments are found in irrigation fluids and medicaments used between visits. Haapasalo found that the irrigation procedure is a key part of a successful root canal treatment as it fulfills several functions, including mechanical, chemical, and microbiological functions. Irrigation is the only way to impact the areas of the root canal that are not touched by mechanical instrumentation, such as oval or narrow root canals, lateral canals, accessory canals, curvatures, irregular root canals, *fins, cul-de-sacs*, and isthmus. These areas have dead pulp tissues and biofilm residues that can only be removed using irrigation fluid [6]. Chlorhexidine (CHX) at a 2% (2% CHX) concentration is the preferred method for eliminating *E. faecalis* and is often used in irrigation procedures [7]. CHX has an antibacterial effect on both Gram-negative and Gram-positive bacteria and its substantivity properties have a long-term therapeutic effect. However, various concentrations of CHX may cause the apoptosis and necrosis of fibroblast cells, epidermal, dermis, and subcutaneous tissue, and it may decrease the number of osteoblast cells [8,9].

Several previous studies have suggested that N-acetylcysteine NAC has an antibacterial effect on endodontic pathogenic bacteria. NAC is not an antibiotic compound, but it is an antioxidant containing thiol groups that effectively reduce the extracellular production of polysaccharides, destroying mature biofilms in the same way as a mucolytic agent and reducing bacterial adhesion [10]. NAC is available in capsules of 200 mg with a pH of 2.5 and as an injectable/inhalation drug at 100 mg/ml with a pH of 7 [11].

NAC is an antioxidant because it plays a role in the donor L-cysteine needed in the synthesis of glutathione (GSH). GSH is an intracellular tripeptide antioxidant that effectively reduces the oxidizing effects of lipids, proteins, and nucleic acids from free radicals, peroxides, and heavy metals [12]. NAC can also increase osteogenesis in bone regeneration [13]. NAC may also prevent tumor necrosis factor-α, which may cause L929 fibroblast death [14].

Quah *et al.* examined NAC as a medicament material and found that NAC is more effective at killing *E. faecalis* ATCC 29212 in biofilms at concentrations of 50 mg/ml and a pH of 11 than calcium hydroxide during a 7–21 days period [15]. Darrag examined NAC's effectiveness as an irrigation material and stated that 200 mg/ml NAC is more effective in killing *E. faecalis* ATCC 29212 and *S. mutans* ATCC 25175 than 5.25% NaOCl and 2% CHX. This study did not mention the NAC pH value and used a long exposure time of 5 min [16]. Cindy *et al.* also found that

200 mg/ml NAC had a good antibiofilm effect on bacteria in the oral cavity [17].

Based on the different concentrations, pH, and application times used in the previous research, this study will compare the antibacterial potential of 200 mg/ml NAC at a pH of 2.5 and a pH of 11 (200 mg/ml NAC pH 2.5 and 200 mg/ml NAC pH 11, respectively) to that of 2% CHX on the post-endodontic clinical isolates of the *E. faecalis* biofilm with 1 min application time identical to that used in clinical irrigation procedures.

METHODS

The *E. faecalis* bacteria used in this study were clinical isolate bacteria extracted from non-vital root canals with periapex abnormalities in patients at the Dental Conservation Specialist Hospital of the Dentistry Faculty, University of Indonesia [18]. *E. faecalis* suspension from the clinical sample culture in the brain heart infusion (BHI) broth was poured into flat bottom 96-well plates. Next, the bacterial suspensions in well plates were incubated at 37°C for 24 h to grow the biofilms.

The 200 mg/ml NAC pH 2.5 solution was made by dissolving 2g NAC in 10 ml distilled water. The 200 mg/ml NAC pH 11 solution was made using the titration method, where 2g of NAC was being added with sodium hydroxide (NaOH) 4M using a pipette while the solution's pH change was measured using a pH meter until it reached a pH of 11. Distilled water was then added to reach a 10 ml solutions.

After incubation, test materials (200 mg/ml NAC pH 2.5, 200 mg/ml NAC pH 11, and 2% CHX) were added to the 96-well plates for 1 min. One well plate as a negative control group was added with a sterile saline solution. Then, all plates were drained and rinsed with a Phosphate Buffer Saline (PBS) solution. The biofilms at the bottom of the plate were then scraped with the tip of a pipette and fed into the PBS solution. The biofilms in the PBS solution were diluted up to 5X and vortexed (mixed using a vortex mixer) for 20 s. Then, the mixture was added to a culture made of BHI agar/gel medium and incubated for 24 h at 37°C. The colonies grown in the medium were counted using colony forming units (CFU).

Data on the total number of *E. faecalis* colonies in the culture were analyzed statistically using SPSS 20.0 software. Shapiro-Wilk and homogeneity tests were conducted, followed by a one-way ANOVA test. The *post-hoc* Bonferroni test was conducted if the one-way ANOVA test yielded a value of $p < 0.05$.

RESULTS

The number of clinical isolates of *E. faecalis* colonies may indicate the antibacterial potential of a test material, where fewer colonies indicate greater antibacterial potential. The highest number of *E. faecalis* clinical isolates was found in the 200 mg/ml NAC pH 2.5 test group (Table 1), i.e., 1128 CFU/ml. This number was still lower than the negative control group, i.e., 1664 CFU/ml.

The lowest mean colony count value was found in the 200 mg/ml NAC pH 11 group (752 CFU/ml). Therefore, the 200 mg/ml NAC pH 11 group had the greatest antibacterial effect on the *E. faecalis* clinical isolates biofilm. The number of *E. faecalis* clinical isolates colonies in the 2% CHX group was lower than the number of colonies in the 200 mg/ml NAC pH 2.5 group, but the number of colonies was still higher in the 2% CHX group than the number found in the 200 mg/ml NAC pH 11 group. The table also shows that at least two groups had a statistically significant different colony count ($p < 0.05$).

The *post-hoc* analysis in Table 2 shows a statistically significant difference in the antibacterial effects of the NAC pH 2.5 groups and the NAC pH 11 group ($p = 0.009$).

Similarly, the NAC pH 11 and 2% CHX groups had statistically significant differences in their colony counts ($p = 0.018$).

Table 1: Average number of the antibacterial effect on the clinical isolates of the *E. faecalis* biofilm (CFU/ml)

Test group	n	Average number of colonies±SD	Value of p
200 mg/ml NAC pH 2.5	3	1128±82	
200 mg/ml NAC pH 11	3	752±22	
2% CHX	3	1078±44	0.006•

•Test one-way ANOVA, $p < 0.05$. CFU: Colony forming units, SD: Standard deviation, *E. faecalis: Enterococcus faecalis*

Table 2: Significant values of the clinical isolate of the *E. faecalis* among the 200 mg/ml NAC at pH 2.5, 200 mg/ml NAC at pH 11, and 2% CHX groups

	NAC pH 2.5	NAC pH 11	CHX 2%
NAC pH 2.5	-	0.009•	1.000
NAC pH 11	0.009•	-	0.018•
2% CHX	1.000	0.018•	-

•Test *post-hoc* bonferroni, $p < 0.05$. *E. faecalis: Enterococcus faecalis*, CHX: Chlorhexidine

DISCUSSION

Clinical isolates of *E. faecalis* bacteria are believed to be more representative of persistent bacteria in root canals than *E. faecalis* ATCC cultures that have been grown in environmentally and nutritionally adjusted conditions [4]. *E. faecalis* can grow and produce biofilms in various media, including polystyrene and PVC. The *E. faecalis* biofilm in this study was grown in 96-well plates, which are flat-bottomed, cylindrical containers made of polystyrene. These well plates can be used to form static biofilms that are similar to biofilms formed in the root canal, which is a closed environment, not a dynamic system, such as the environment surrounded by the bloodstream. The biofilms formed on well plates also share the same characteristics as mature biofilms, which are tolerant to antibiotics and resistant to the immune system [19].

After their formation, the biofilms in this study were exposed to antibacterial agents for 1 min; this is the exact irrigation time a sonic machine uses [20].

This study used 200 mg/ml NAC at a pH of 2.5, which is readily available in the form of capsules, in accordance with Cindy *et al*. and Darrag. The 200 mg/ml NAC pH 2.5 treatment was compared with 200 mg/ml NAC with a pH of 11. PH 11 was chosen based on Quah *et al*., who found it to be a more effective pH for the NAC than lower pH levels. The study also used 2% CHX as a positive control because it is the preferred antibacterial agent and is commonly used in irrigation procedures in cases of persistent endodontic infection.

The different antibacterial capabilities of these three materials are described in research conducted by Quah *et al*. The NAC pH 2.5 and NAC pH 11 test groups had statistically significant differences in their colony counts. The comparison of these two groups clearly showed the effect of pH changes on the antibacterial potential of NAC because NAC pH 11 had higher antibacterial potential than NAC pH 2.5.

Thiol groups have a sulfur and a hydrogen atom attached by a strong covalent bond. When the NAC pH is increased through the addition of NaOH, the hydroxyl group (-H) derived from NaOH releases the covalent bond and produces a reactive thiolate anion (-S-). This thiolate anion is more effective in breaking the protein disulfide bond in the biofilm. The breakdown of the disulfide bond in the three-dimensional structure of the *E. faecalis* clinical isolate biofilm facilitates and makes it easier for the active agent NAC to bind with the thiol-disulfide groups of bacteria in the biofilm, which may lead to the intermolecular and intramolecular destruction of both the bacterial wall protein and the

molecules produced by the bacteria. It will also affect the virulence of the *E. faecalis* clinical isolate bacteria.

In this study, when NAC pH 2.5 was also compared with 2% CHX. However, the results did not support the research conducted by Darrag since the antibacterial potential of NAC pH 2.5 was lower than that of 2% CHX. This difference in outcomes may be due to the different research methodology used, such as the difference between the exposure times in each study. Darrag found that the antibacterial potential of 200 mg/ml NAC pH 2.5 was greater than the CHX when an exposure time of 5 min was used, which may indicate that thiol groups in the 200 mg/ml NAC pH 2.5 require a longer time to break down the disulfide bonds in the biofilm proteins than the electrostatic velocity of the positive charge of CHX to bind to the negative surface of the bacteria through water channel biofilms. However, the difference was not statistically significant.

The low number of colonies remaining in the NAC pH 11 group compared to the 2% CHX group indicates that the antibacterial potential of NAC pH 11 was greater than that of 2% CHX, which supports the research of Quah *et al*. NAC's ability to directly damage the three-dimensional structure of the biofilm enables the antibacterial to more easily penetrate the biofilm and damage the intermolecular and intramolecular bacterial wall proteins. CHX's antibacterial effect is the result of cytoplasmic precipitation. In 1 min treatment, the CHX molecules were able to bind electrostatically to the bacterial wall through the biofilm water channel, but the reaction speed is lower and it damages the structure of the biofilm differently than NAC pH 11.

CONCLUSION

NAC has antibacterial potential against *E. faecalis* clinical isolates biofilm. The antibacterial potential of 200 mg/ml NAC pH 11 is greater than that of 200 mg/ml NAC pH 2.5 and 2% CHX, whereas 200 mg/ml NAC pH 2.5 and 2% CHX have the same antibacterial potential.

ACKNOWLEDGMENT

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