

# The Differential Effectiveness of Calcium Hydroxide (Ca(OH)<sub>2</sub>) Combination with Chlorhexidine Digluconate (CHX) 2% and Clindamycin Hydrochloride 5% Against *Enterococcus faecalis* (in vitro)

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## ABSTRACT

**Background:** The main factor causing failure of root canal treatment is re-infection of the bacterium *Enterococcus faecalis*, so it is necessary to give the right intracanal medicament. Calcium hydroxide (Ca(OH)<sub>2</sub>) is an intracanal medicament often used in endodontic treatment. To increase the effectiveness of calcium hydroxide, a mixing agent is needed. Mixing agents such as Chlorhexidine digluconate (CHX) 2% (aqueous) and Clindamycin hydrochloride 5% (viscous). The viscous mixing agent can penetrate well into the dentinal tubules, and the antibiotic can fight various root canal endodontic pathogens and dentinal tubules without causing discoloration. This study aims to determine the differential effectiveness of calcium hydroxide combination of chlorhexidine digluconate 2% and Clindamycin hydrochloride 5% as an intracanal medicament against *Enterococcus faecalis* (in vitro).

**Methods:** The design was in vitro experimental laboratories that tested the value of turbidity (Optical Density) using a spectrophotometer before and after being treated. Data were analyzed by the Kruskal-Wallis test and then Mann-Whitney U Test.

**Results:** The positive control group had the largest negative Optical Density (OD) difference, followed by a calcium hydroxide combination of Chlorhexidine digluconate 2%, and the third sequence was a calcium hydroxide combination of Clindamycin hydrochloride 5%. A negative OD difference indicates a decrease in the number of bacterial cells.

**Conclusion:** There are differences in the effectiveness of the antibacterial of intracanal medicament calcium hydroxide combination of Chlorhexidine digluconate 2% and calcium hydroxide combination of Clindamycin hydrochloride 5%.

**Keywords:** Calcium hydroxide (Ca(OH)<sub>2</sub>), Chlorhexidine digluconate (CHX) 2%, Clindamycin hydrochloride 5%, *Enterococcus faecalis*.

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## INTRODUCTION

The risk of treatment failure increases if microorganisms grow and colonize the dentine tubules despite irrigation of the root canal.<sup>1</sup> The prevalence of *Enterococcus faecalis* bacteria in root canal treatment failure ranges from 24% to 77%.<sup>2</sup> As the *Enterococcus faecalis* bacteria can withstand extreme environments and very alkaline hydrogen (pH) potential, and high salt concentrations.<sup>3</sup> Giving the right medicament material is needed to reduce the number or kill root canal bacteria to prevent reinfection.<sup>4</sup>

Calcium hydroxide (Ca(OH)<sub>2</sub>) is a root canal medicament material frequently

used in the endodontic treatment for many years with alkaline pH; thus, it has an anti-microbial effect.<sup>5</sup> Calcium hydroxide is considered less effective in killing *Enterococcus faecalis* because *Enterococcus faecalis* has a proton pump that maintains the pH balance, making the bacteria survive in an alkaline environment.<sup>3</sup> To increase calcium hydroxide (powder) effectiveness in killing *Enterococcus faecalis*, a medicament vehicle is needed to increase calcium hydroxide and facilitate attachment into the root canal.<sup>4</sup>

The aqueous vehicle can quickly decompose Calcium (Ca) and Hydroxyl (OH<sup>-</sup>) ions and facilitate dissolution.<sup>6</sup> A viscous vehicle is a water-soluble substance

that slowly releases Calcium ions and Hydroxyl ions for an extended period of time. This material is recommended as the paste can remain in the root canal for extended periods.<sup>7</sup> According to Cruz et al. (2002), viscous vehicles reach the apical foramen faster than liquid mixing agents. The area and depth of penetration of the viscous mixing material are significantly greater than that of the liquid mixing agent. The high surface tension of the liquid mixing material can inhibit penetration into the dentinal tubules. It will benefit from thick mixers with lower surface tension as they can penetrate well into the dentinal tubules.<sup>8</sup>

Chlorhexidine digluconate (CHX)

is an aqueous medicament vehicle.<sup>9</sup> Chlorhexidine digluconate is a material with broad-spectrum anti-microbial power and can maintain its anti-microbial power for a long time.<sup>10</sup> Calcium hydroxide with Chlorhexidine digluconate paste can eliminate more persistent microorganisms, such as *Enterococcus faecalis*, since it can function as a physical barrier in the root canal.<sup>11</sup> Chlorhexidine digluconate 2% is bactericidal, which is effective in killing bacteria *Enterococcus faecalis* and *Candida albicans*.<sup>12</sup>

Another mixing ingredient that can help kill the bacteria *Enterococcus faecalis* comes from the antibiotic group, namely Clindamycin, as it is a drug of choice in odontogenic infections. The antibiotic can fight various anaerobic, facultative, and anaerobic bacteria.<sup>13</sup> Clindamycin antibiotics that are active in vitro are Clindamycin hydrochloride.<sup>14</sup> Clindamycin is effective against various endodontic pathogens in root canals and dentinal tubules and does not cause discoloration.<sup>15</sup> The mechanism of action of Clindamycin hydrochloride is to inhibit peptide bonds formed from bacterial Deoxyribonucleic Acid (DNA) that cause cell death.<sup>16</sup>

This study aims to determine the differential effectiveness of calcium hydroxide combination of chlorhexidine digluconate 2% and Clindamycin hydrochloride 5% as an intracanal medicament against *Enterococcus faecalis* (in vitro).

## MATERIAL AND METHODS

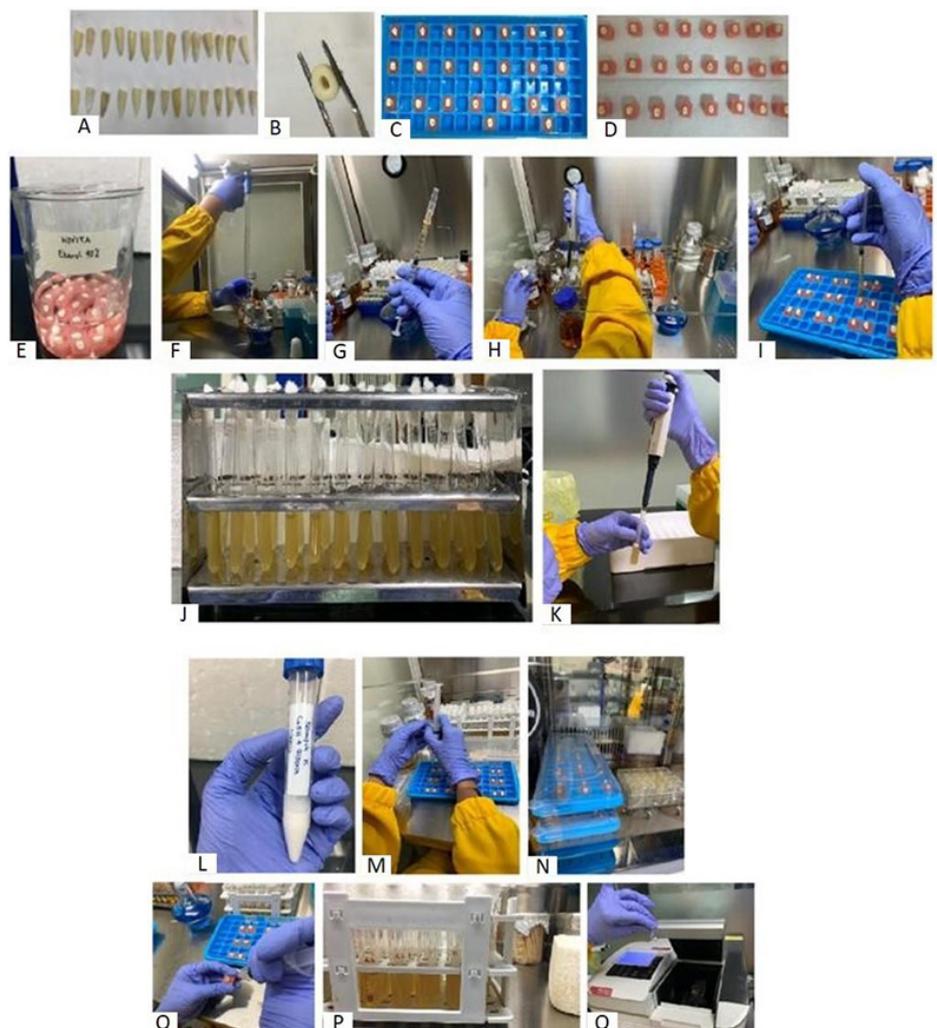
This research design is in vitro laboratory experimental of intracanal medicament on *Enterococcus faecalis*. Each group consisted of 2% chlorhexidine digluconate (CHX) with calcium hydroxide (group A), clindamycin hydrochloride 5% with calcium hydroxide (group B), glycerin 60% combination of calcium hydroxide (group C), glycerin 100% (group D as a control) each received six repetitions. The variable affected in this study was the growth of *Enterococcus faecalis* clinical bacteria in the tooth's root canal.

This research was conducted at the Molecular Medicine and Therapy Research Laboratory. The research was carried out from January to April 2020.

The steps were as follows: first, measuring the working length of 24 permanent, single-rooted permanent premolar teeth, and root canal preparation was later performed. After that, the prepared teeth were cut using a diamond disc in the Cementoenamel Junction (CEJ) section, followed by planting the acrylic self-cure teeth (Hylon®, England) using a mold and coding each treatment group using a label. The teeth were sterilized using a 90% ethanol solution in a beaker, covered with aluminum foil, and allowed to stand

for 12 hours at room temperature. It was then put into an autoclave (Hirayama®, Tokyo, Japan) with a temperature of 121°C for 15 minutes of sterilization with 1 atm pressure. It was then removed and froze (Figure 1).

Master suspension of *Enterococcus faecalis* bacteria on Brain Heart Infusion (BHI) broth was carried out by inserting 2 ml BHI broth and 1 ml suspension of *Enterococcus faecalis* bacteria into the test tube. The test tube (LabWare®, United States of America) was closed



**Figure 1.** (A) Teeth after cutting using diamond disc; (B).Root canal after preparation; (C &D). Teeth in acrylic self-cure (Hylon®) using a mold; (E). Sterilization of teeth on ethanol 90%; (F).Making a suspension of *Enterococcus faecalis* bacteria; (G).*Enterococcus faecalis* bacterial suspension; (H).Brain Heart Infusion (BHI) is inserted into the test tube; (I).Teeth Incorporation; (J).Measurement of Optical Density (OD) value before treatment (spectrophotometer); (K).The results of incubation before for 24 hours; (L). Calcium hydroxide (Ca(OH)<sub>2</sub>) with glycerine 60%; (M).Application of medicaments; (N).Swab the root canal with a paper point; (O).Irrigation of root canal after 72 hours incubation; (P).The result is incubation after 24 hours; (Q).Optical Density (OD) after calculation (spectrophotometer).

using cotton and incubated at 37°C for 24 hours. Furthermore, the incorporation was carried out by entering the suspension of *Enterococcus faecalis* 0,05 ml into the root canal using a tuberculin syringe and incubating at 37°C for 24 hours. The teeth were then removed and left until it was moist.

Measurement of Optical Density (OD) value was conducted before using a spectrophotometer (Dynamica®, Livingston, United Kingdom) to determine the number of bacterial populations in the root canal before being treated. The root canals were cleaned using the same size paper point by inserting BHI into a test tube. Furthermore, the paper point was inserted into a test tube containing BHI. All sample groups were vortexed (Scilogex®, Rocky Hill, United States of America) and incubated at 37°C for 24 hours. After that, the BHI was taken out containing the paper points from the incubator (Labtech, Gyeonggi-do, South Korea) and then vortexed. BHI was taken using a micropipette and put into a disposable cuvette (Brand®, Essex, United States of America). The cuvette was inserted into the spectrophotometer to obtain the OD value.

The group A medicament materials were mixed by inserting 2 grams of calcium hydroxide (hydroxido calico PA biodinamica®) and 1.5 ml of liquid chlorhexidine digluconate (clorexoral 2% biodinamica®, ibipora, Brazil) into the test tube, and it was vortexed while stirring using an excavator until evenly mixed and forming a paste consistency. The paste was then put into a syringe. Besides, the group B medicament materials were mixed by inserting 0.15-gram clindamycin hydrochloride and 3 ml of 60% glycerin into the test tube and vortexed while stirring using an excavator until it was homogeneous. The preparation was later taken as much as 2 ml into a test tube and added calcium hydroxide powder as much as 2 grams. It was then vortexed while stirring using an excavator until it was well blended and formed a paste consistency. The paste was put into a syringe. Furthermore, the group C medicament materials were mixed by entering 2 grams of calcium hydroxide powder and 60 ml of glycerin as much as 1.5 ml into a test

tube. It was diverted while stirring using an excavator until it was evenly mixed, and a paste consistency was formed. The paste was then put into a syringe. The group D medicament materials were mixed by adding 100% glycerin to the syringe.

The application of medicament material to the root canals using a syringe and combined with lentulo was then incubated for three days or 72 hours at 37 °C in an incubator. After the incubation, the medicament material in the root canal was irrigated with an irrigation syringe using a 0.9% saline solution of 20 ml. The root canal was dried using a sterile paper point consistently from coronal to apical for 10 seconds. The paper point was inserted into the BHI broth's test tube and then vortexed (Figure 1). After that, it was incubated at 37°C for 24 hours. After using a spectrophotometer, the measurement of OD value after applying medicament material was carried out by taking BHI using a micropipette and putting it into a disposable cuvette. The cuvette was

inserted into the spectrophotometer to obtain the OD value. Analysis of the data used is the Kruskal-Wallis and Mann-Whitney U test to identify how much difference was in each group's effectiveness of antibacterial power.

## RESULTS

The study results have been obtained by calculating the difference in Optical Density (OD) values between pre and post-application of medicament material. A negative OD difference indicated a decrease in the number of bacterial cells after 72 hours of incubation. In comparison, a positive OD difference indicated an increase in bacterial cells after 72 hours of incubation. The results of the OD value before and after the application of medicament material to the root canal can be seen in Figure 2. The graph shows that the calcium hydroxide combination of Chlorhexidine digluconate 2% has a more excellent OD value than the calcium

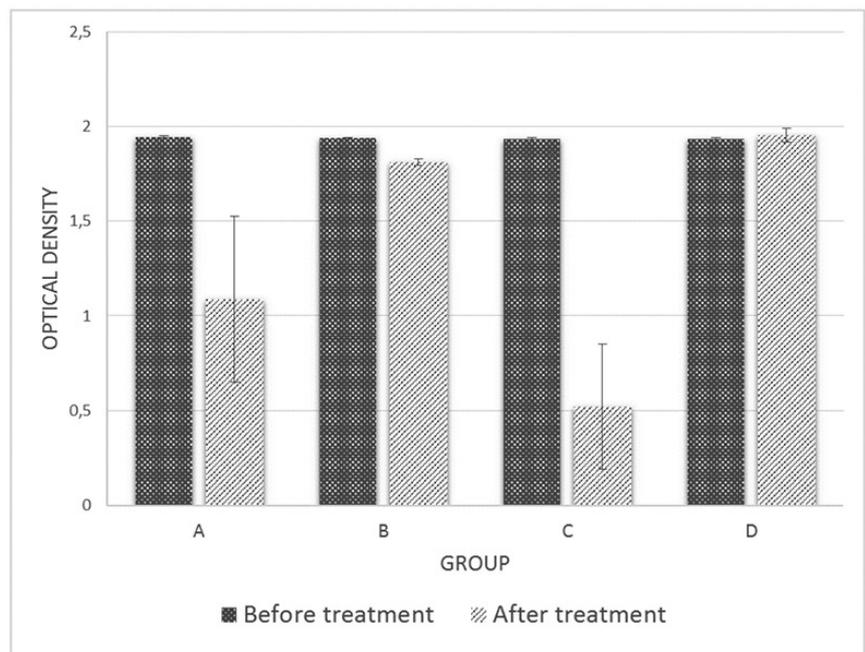


Figure 2. Average of OD Value before and after treatment in every group

Table 1. Kruskal Wallis statistical test results

Intervention group	Mean Rank
Ca(OH) <sub>2</sub> + CHX 2%	13.00
Ca(OH) <sub>2</sub> + Clindamycin HCL 5%	12.83
Ca(OH) <sub>2</sub> + Glycerin 60%	5.00
Glycerin 100%	19.17
<b>Asymp. Sig.</b>	<b>0.007</b>

**Table 2. Mann-Whitney U test statistical results**

(I) Group	(J) Group	Asymp. Sig. (2-tailed)
Ca(OH) <sub>2</sub> + CHX 2%	Ca(OH) <sub>2</sub> + Clindamycin HCL 5%	1.000
	Ca(OH) <sub>2</sub> + Glycerin 60%	.150
	Glycerin 100%	.337
Ca(OH) <sub>2</sub> + Clindamycin HCL 5%	Ca(OH) <sub>2</sub> + Glycerin 60%	.004*
	Glycerin 100%	.010*
Ca(OH) <sub>2</sub> + Glycerin 60%	Glycerin 100%	.004*

\*p&lt;0.05

hydroxide combination of Clindamycin hydrochloride 5%. The lowest OD difference value lies in the negative control, glycerin 100%. The researchers examined the hypothesis on the difference in OD of each group using the non-parametric Kruskal Wallis test as there were groups with abnormal data distribution (Table 1).

The statistical test result obtained a significance value of 0.007, which indicated a p<0.05. It revealed a difference in the effectiveness of group A and group B as an intracanal medicament against *Enterococcus faecalis* (in vitro). A further test was carried out, namely the Mann-Whitney U test, to find out the groups that significantly differed in the effectiveness of antibacterial power.

The result of the Mann-Whitney U test indicated that the calcium hydroxide group combination of Chlorhexidine digluconate 2% and calcium hydroxide combination of Clindamycin hydrochloride 5% did not differ significantly (Table 2; p > 0.05).

## DISCUSSION

The study showed that out of six samples, the calcium hydroxide combination of Chlorhexidine digluconate 2% was obtained in three samples showing a significant decrease in the number of bacteria. In contrast, the other three samples experienced a slight increase in the number of *Enterococcus faecalis*. The decrease in the number of bacteria was likely to occur since the combination of Calcium hydroxide and Chlorhexidine digluconate would increase each material properties' synergism due to

its reactive oxygen and deprotonation.<sup>12</sup> Deprotonation of Chlorhexidine digluconate could increase the viscosity of Calcium hydroxide (Ca(OH)<sub>2</sub>) paste; thus, Calcium (Ca) paste calcium hydroxide was no longer in contact with the root canal wall.<sup>12</sup> Chlorhexidine digluconate also had broad-spectrum anti-microbial activity against various microorganisms, including *Enterococcus faecalis*.<sup>17</sup> At low concentrations (0.12%-0.2%), they were bacteriostatic. Meanwhile, at high concentrations (1.8-2%), they were bactericidal, causing deposition of bacterial cytoplasm and cell death.<sup>17</sup> The reduction of effectiveness of a mixture of liquid Chlorhexidine digluconate and calcium hydroxide could be caused by deprotonation of biguanide on > 8. Thus, it also reduced solubility, which could inhibit interactions with negatively charged bacterial cell membranes.<sup>18</sup>

The calcium hydroxide group combined with 5% Clindamycin hydrochloride obtained from all samples decreased the insignificant number of bacteria. The decrease in the number of bacteria is possible as Clindamycin is bacteriostatic, which inhibits bacterial protein.<sup>19</sup> It is in line with the study by Surender et al., which states that Clindamycin was effective against *Enterococcus faecalis* after 24 hours of incubation in agar media.<sup>20</sup> When Clindamycin and calcium hydroxide is combined, calcium hydroxide acts on the bacterial cell membrane, and Clindamycin acts synergistically in the cell. The study of Surender et al. also examined the effectiveness of chlorhexidine gel. Being compared to Clindamycin

1% calcium hydroxide combination, chlorhexidine gel had a more significant inhibition zone.<sup>20</sup> On the other hand, the research conducted by Molander and Dahlen showed that Clindamycin combination saline did not provide more advantages than conventional intracanal medicaments, such as calcium hydroxide. However, no negative controls were used, and the concentration was not mentioned. The ability of Clindamycin to penetrate the root canal system was also not investigated. Nonetheless, Clindamycin paste could eliminate bacterial growth in 21 of the 25 teeth tested on the 14th day. In the remaining four teeth, *Enterococci* was the dominant species.<sup>21</sup>

Based on figure 2, in the 60% glycerin combination with calcium hydroxide as a positive control group, all samples experienced a significant decrease in the number of bacteria. The graph of the median value of the intracanal medicament group found that the 60% glycerin calcium hydroxide group had the most considerable median optical density (OD) different than the other three groups, which was 1.924. It is consistent with the results of research by Dewi et al., which stated that a mixture of calcium hydroxide with glycerin showed the highest inhibitory zones in *F. nucleatum* and *E. faecalis*.<sup>22</sup> Glycerin can increase the penetration of medicament into the dentinal tubules and the anti-microbial activity of the medicament.<sup>22</sup> The high glycerin alkalinity causes an increase in the potential of hydrogen (pH), and the hygroscopic properties of glycerin play a role in releasing calcium hydroxide ions gradually over a more extended period.<sup>22</sup>

Many factors affected the process and the result of this study due to limitations in the research, including the tool's limitations where the thickness and homogeneity of the paste of the medicament material were only based on the subjectivity of the researcher without being measured using a tool. Thus, it was possible to influence the effectiveness of the material studied. In addition, the tools used might not be completely free from other bacteria. The occurrence of other bacterial contamination can increase the test material's turbidity value when the bacteria also develops on Brain Heart

Infusion (BHI) media. Furthermore, the study in each medicament group to eliminate root canal bacteria is not optimal due to inadequate root canal cleansing, which only used a 0.9% saline solution.<sup>23</sup> Inadequate cleaning results in the possibility of remaining calcium hydroxide residues from the root canal wall surface.<sup>23</sup> Calcium hydroxide residues in the root canal can make the material not entirely effective against several endodontic pathogens, including the species *Enterococcus faecalis*, which leads to various incidents of re-infection or flare-ups.<sup>23</sup>

According to Deepak et al. (2015), using appropriate mechanical instrumentation combined with chelating agents and irrigation using sodium hypochlorite will enable the cleanliness of the walls of the adequate root canal.<sup>24</sup> Agitation of irrigation materials with mechanical devices can increase the efficiency of removing intracanal medicament.<sup>24</sup>

## CONCLUSION

Based on the result of this research, it can be concluded that there was a difference in the effectiveness of the antibacterial power of intracanal medicament calcium hydroxide combination of 2% Chlorhexidine digluconate and calcium hydroxide combination of 5% Clindamycin hydrochloride.

Further research is needed on the effects of combining glycerin with clindamycin hydrochloride with a longer incubation time and other testing methods. It is necessary to uniform the thickness of each medicament material and adequate irrigation. In addition, it is recommended to conduct the same testing method on the combination of intracanal medicament with other medicament vehicles to increase the antibacterial power against *Enterococcus faecalis*.

## ETHICAL CLEARANCE NUMBER/ STATEMENT:

This research has been approved by Health Research Ethics Committee, Faculty of Medicine and Health Sciences, Universitas Muhammadiyah Yogyakarta with the ethical exemption letter No. 029/EC-EXEM-KEPK FKIK UMY/XII/2019.

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There is no conflict of interest. We have not published this article yet before.

## AUTHOR CONTRIBUTIONS:

The first author contributed to establishing concepts, design, definition of intellectual content, literature search, clinical studies, experimental studies, data analysis, manuscript preparation, editing, and review. The second author contributed to establishing the definition of intellectual content, literature search, experimental studies, data acquisition, data analysis, and statistical analysis.

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