Antibacterial activity of various calcium hydroxide solvents against *Fusobacterium nucleatum* and *Enterococcus faecalis*

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Antibacterial activity of various calcium hydroxide solvents against *Fusobacterium nucleatum* and *Enterococcus faecalis*

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Abstract. *Fusobacterium nucleatum* and *Enterococcus faecalis* are the most common types of bacteria found in root canal failure. Various ways are done to eliminate such pathogens, one of them by adding various solvents to increase the antibacterial activity of intracanal calcium hydroxide medication. The purpose of this study was to determine the antibacterial activity of various calcium hydroxide solvents against *Fusobacterium nucleatum* and *Enterococcus faecalis*. The antibacterial activity of chlorhexidine 2%, glycerin, povidone-iodine 2% and distilled water (control group) as calcium hydroxide solvents were tested in vitro. Zone of inhibition of solvents was observed. The results showed that the remarkable inhibition of the bacterial growth was shown by adding glycerin solvent to calcium hydroxide. This activity was due to its ability in increasing alkaline activity of calcium hydroxide. Hence, glycerin solvent of calcium hydroxide can be used to increase antibacterial activity against root canal microbes.

1. Introduction

Calcium hydroxide is widely used as root canal materials in dentistry [1]. This medicament has ability to eliminate bacteria which can not be destroyed only by instrumentation and irrigation processes [2]. Calcium hydroxide possess antibacterial properties and remineralization capabilities in dentin [3]. It also has ability in accelerating the healing of periapical lesions. Several studies had been reported that calcium hydroxide is very effective as intracanal medicaments and being indicated for several clinical conditions [4].

The failure of root canal treatments is associated with the persistence of microbial infection in the root canal system with or without periradicular area [5]. Residual bacteria and their byproducts remaining in root canal system or coronal leakage cause the endodontic failure [6]. The most common types of bacteria found in root canal failures are *Fusobacterium nucleatum (F. nucleatum)* and *Enterococcus faecalis (E. faecalis)* [7,8].

Fusobacterium nucleatum is Gram-negative bacteria with small spindle-shaped rod and associated with some dental diseases, such as dental abscess, periapical disease and failed root canal treatment [9]. The pathogenicity of *F. nucleatum* is related to its virulence and ability to survive in root canal

system. It also produces poly-gamma-glutamate, that has a role in virulence and in survival in some unfavorable conditions [10]. Its pathogenicity depends on the degree of anaerobiosis, pH level, the availability of exogenous and endogenous nutrients. The use of calcium hydroxide is limited, and still remains a predominance of obligate anaerobic bacteria, including species of *Fusobacterium* [11,12].

Enterococcus faecalis is a coccus Gram-positive bacteria, facultative anaerobe that commonly found in failed root canal treatment [13]. *E. faecalis* is able to withstand nutrient depletion, capable to suppress lymphocyte and form biofilm [14]. Previous study reported that microflora with high prevalence of persistent infections of root canal system is *E. faecalis* [15]. Chai stated that *E. faecalis* is resistant to the administration of calcium hydroxide [16]. This condition is due to its ability to maintain the pH balance, as a result of ion penetration to cell membrane and the capacity of cytoplasmic buffer. Thus *E. faecalis* still remains in root canal system [17]. It has lipotheic acid (LTA), a virulence factor, playing a role in root canal infection. LTA is released from cell lysis and binds to target cells, interacts with cell immune systems and finally causes damage [18]. This bacteria produce pathological changes either directly through the productions of toxins or indirectly by inducing the inflammatory process [19].

Various methods are utilized to eliminate bacteria that cause failed root canal treatment and persistent perirardicular lesions. One of them is by combining root canal medicament with solvents which has antibacterial properties. Antibacterial agents in root canal system commonly used as irrigation in endodontic treatment are chlorhexidine, povidone-iodine, glycerin and combine with distilled water irrigation as canal rinse. Mohammadi reported that chlorhexidine had antibacterial activity because of its interaction with phospholipids and lipopolysaccharides on the bacterial cell membrane [20]. Kanagalingam resumed that povidone-iodine had rapid antimicrobial activity against highly resistant *E. faecalis*. Its activity was due to its modes of action on multiple pathogenic targets [21]. Glycerin exhibits strong bactericidal activity against Gram-positive and Gram-negative bacteria [22]. Distilled water does not have antibacterial activity, but it uses for cleaning the root canal from debris and medicament remnants.

Antibacterial activity of calcium hydroxide mixed with various solvents has not yet been performed. Therefore, this study presented antibacterial properties of various solvents added to intracanal medicament of calcium hydroxide.

2. Methods

The study was in vitro experimental laboratories. It was conducted on Province's Health Laboratory of South Sumatera, Palembang, Indonesia.

2.1. Preparation of calcium hydroxide

Calcium hydroxide (Sunway, Jiangsu, China) purchased from Cobra Dental Supply, Yogyakarta, Indonesia was measured with digital scales of 8 g and divided into 8 groups then placed in glass beakers. Group 1 was to observe the inhibition zone of *Fusobacterium nucleatum* ATCC 23726 (obtained from Province's Health of South Sumatera Laboratory), while group 2 was to evaluate the inhibition zone of *Enterococcus faecalis* ATCC 29212 (obtained from Province's Health of South Sumatera Laboratory). Each groups was divided into 4 subgroups (A,B,C,D) with different solvents. Group A was 0.2% chlorhexidine (Promedraharjo Farmasi, Indonesia), group B was glycerin solvent (Promedraharjo Farmasi, Indonesia), group C was povidone-iodine (PT. Mahakam Beta Farma, Indonesia), and group D was distilled water (Bratachem, Indonesia) as control. All solvents were mixed with calcium hydroxide with ratio of 1:1 until it reached the consistency like pasta.

2.2. Preparation of standard inoculum

The colonies of *F. nucleatum* and *E. faecalis* were emulsified with BHIB media in test tubes, incubated at 37^0C for 48 hours anaerobically, then adjusted to 0.5 Mc Farland standards (10^8 CFU ML^{-1}). The suspension was diluted to get the infection dose of *F. nucleatum* (1 x 10^8 CFU/ml) and *E. Faecalis* (1 x 10^6 CFU/ml) [23].

The antibacterial activity against the growth of *F. nucleatum* and *E. faecalis* was evaluated using agar diffusion method. Filter paper disc, containing the mixture of calcium hydroxides and various

solvents base on respective groups was placed on agar. After that, all petridiscs were incubated in incubator at temperature of 37^0C for 24 h, anaerobically. The diameters of inhibition growth zones (mm) were determined.

2.3. Statistical analysis
Data were analyzed using one-way ANOVA and Tukey's post hoc test using SPSS 22 vs. (IBM® inc.pvt ltd.). Confidence level was set at $P<0.05$

3. Results
The observation of inhibition zone of *F. nucleatum* and *E. faecalis* in agar after 24 hours showed that four solvents mixed with calcium hydroxide powder had antibacterial activity, signified by clear zone. The mean value of the inhibition zone for each groups was seen in table.1

Tabel 1. Antibacterial activity of various solvents by disc diffusion method.

Bacterial species	N	Inhibition Zone in Diameter (mm)			
		Group A	Group B	Group C	Group D
Fusobacterium nucleatum	8	11.42 ± 0.41	14.23 ± 0.37	12.61 ± 0.59	12.55 ± 1.11
Enterococcus faecalis	8	8.97 ± 0.49	11.78 ± 0.72	10.10 ± 0.33	10.07 ± 0.78

From table 1, it was found that inhibitory zone of both bacteria had the highest mean of group B (calcium hydroxide + glycerin), followed by group C (calcium hydroxide + povidone-iodine), group D (calcium hydroxide + distilled water), and group A (calcium hydroxide + chlorhexidine).

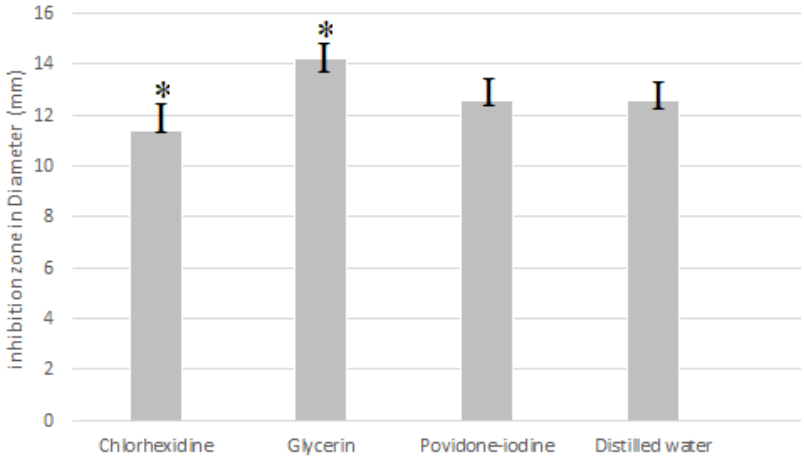

Figure 1. Mean zone of inhibition for *F. nucleatum.* *Post Hoc Tukey's significant test compare to distilled water solvent, p value <0.05.

One way Anova test for group 1 and group 2 showed each p value was 0.000. It meant that all the groups had a significant difference in zone inhibition of *F. nucleatum* and *E. faecalis.* Tukey's Post Hoc test was used to compare the antibacterial activity of all groups. The difference in the mean of diameter inhibition zone for *F. nucleatum* and also *E. faecalis* between chlorhexidine solvent and others; glycerin solvent and others were found to be significant, while there was no significant difference between povidone-iodine and distilled water solvent (figure 1 and figure 2).

4. Discussions
Calcium hydroxide is a root canal medicament that has antibacterial effect. In root canal system, it dissociates into hydroxyl and calcium ions on contact with aqueous solvent, causing the damage of bacterial cytoplasmic membrane, protein denaturation and the inhibition of DNA replication [24]. This

medicament also acts as a barrier in preventing bacterial entrance in root canal system. Hydroxyl affects the viability of anaerobic bacteria. The diffusion of this ion causes alkaline environment that influences bacterial defenses [25]. Calcium hydroxide reported having an ability in eliminating microorganisms, such as *E. faecalis, F. nucleatum, A. naeslundii* and *Candida albicans* by its alkaline stress. It is also effective in removing *E. faecalis* biofilm in root canal system [26,27]. In general, the most commonly use of calcium hydroxide solvent is distilled water. Distilled water has netral pH and good solubility, but does not have antibacterial properties and is not able to affect antibacterial activity [28].

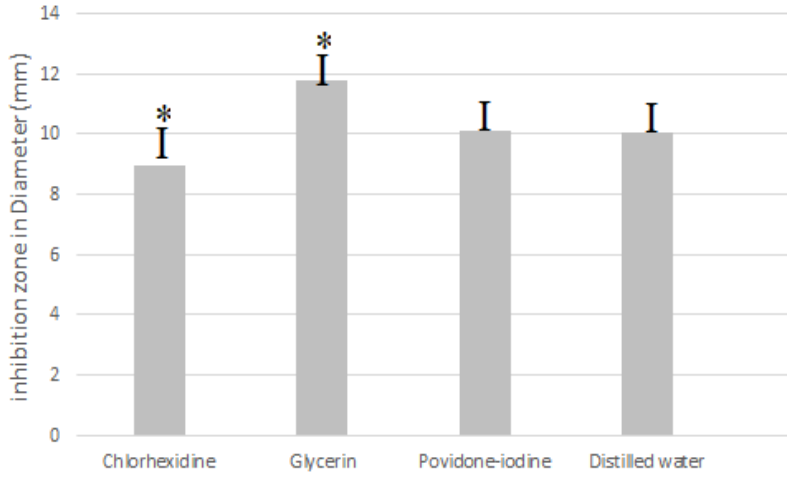

Figure 2. Mean zone of inhibition for *E. faecalis.* *Post Hoc Tukey's significant test compare to distilled water solvent, p value <0.05.

Compare to others, glycerin showed the highest zone of inhibition in both *F. nucleatum* and *E. faecalis.* Glycerin is a trihydric alcohol that has 3 carbon atoms and 3 hydroxyl (OH) atoms and is able to bind to other substances potentially. Recent study reviewed that glycerin worked up medicament penetration into the dentinal tubules and enhanced antimicrobial activity of the drugs [29]. Glycerin contributes in releasing calcium hydroxide ions gradually, by its hygroscopic nature for longer period of time [30]. The high alkalinity of glycerin leads the increase of pH. If the pH in a medium or environment is not optimal (pH=9), it will disrupt enzymes and interfere with the growth of bacteria. *F. nucleatum* grows and multiplies in pH 8-8.3 [31], while *E. faecalis* grows in wide range of pH and resists alkalinity at pH 11.5 [32]. The pH level of calcium hydroxide is 12.5 and the addition of glycerin can increase the pH level [33].

The combination of calcium hydroxide and povidone-iodine solvent showed the same effect as calcium hydroxide and distilled water. Povidone-iodine is widely known as antiseptic that has ability in killing many organisms. Povidone-iodine activity depends on the release of its ions. Iodine reacts with the basic function of N-H contained in amino acids, blocks hydrogen bond and disrupts protein structures. Povidone-iodine changes the physical properties of lipids and leads immobilization of membranes [34]. The antibacterial activity of povidone-iodine solvent is equivalent to distilled water. These findings indicate that the addition of povidone-iodine solvent is unable to improve the antibacterial properties of calcium hydroxide. It is because povidone-iodine has lower pH of 6, so the pH combination of this solvent and medicament is reduced and causes bacteria to grow [35]. However, the antibacterial properties of povidone-iodine still work on both types of bacteria, so that its combination is capable of providing antimicrobial effects. Previous study reported that povidone-iodine suppressed *F. nucleatum* biofilm and reduced 100% *E. faecalis* after 30 minutes [36,37].

Chlorhexidine is most commonly used in dentistry as oral rinse and endodontic treatment. Chlorhexidine inactives microorganisms with broader-spectrum compare to other antimicrobials [38].

Its activity is due to its interaction of the positive charge of binding molecules and the negatively charged phosphate groups on cell walls, causes instability of cell wall, alters osmotic equilibrium, induces leakage and eventually results in a toxic effect. It can easily kill microorganism in short period of time [39]. In root canal treatment, chlorhexidine is used as irrigation and single intracanal medicament.

Chlorhexidine has strong ability in killing *F. nucleatum*. Rathke *et al* evaluated antibacterial activity of chlorhexidine and calcium hydroxide containing gutapercha points and found that chlorhexidine containing gutapercha point was significantly more effective in killing *F. nucleatum* than calcium hydroxide containing gutapercha points [40]. The effectiveness of calcium hydroxide in killing *E. faecalis* is higher than povidone-iodine in infected dentin [37]. Another finding reported that chlorhexidine increased bactericidal action of endodontic medicament more effective and lower opportunity to develop resistance than glycerin [41]. The gel and liquid formulation of chlorhexidine kill *E. faecalis* within 1 minute, while they are also capable of killing Gram-negative bacteria within 15 seconds [42]. Misuriya *et al* represented that chlorhexidine as intracanal irrigation inhibited the growth of *F. nucleatum* and *E. faecalis* with the average zone of inhibition were 25.07 $\pm$ 0.87 mm and 20.85 $\pm$ 0.83 mm respectively [43].

Previous study reported that chlorhexidine was unable to disrupt bacterial biofilm. The efficacy of eliminating *E. faecalis* biofilm was less than sodium hypochlorite [44]. Kanisavaran reviewed that the residual effect of chlorhexidine in root canal system was 48 hours up to 12 weeks. This antibacterial activity depended on the number of the molecules to interact with dentine tubules [45]. Study of Shahani and Reddy revealed that 2% chlorhexidine had prolonged antibacterial substantivity lasting up to 72h. Its effectivity was higher than 1% povidone-iodine because povidone-iodine did not have prolonged antibacterial effect. So that chlorhexidine should be used as final irrigation in root canal system [46].

The combination of calcium hydroxide and chlorhexidine as solvent was used in an attempt to increase antibacterial activity in root canal system. In contrast, the effect of this mixture showed the decrease of antimicrobial power of root canal medication. This condition was related to pH reduction. When this dressing agent is associated, elevated pH levels may cause precipitation of chlorhexidine, since its optimal action pH is 5 [47]. The antibacterial activity of chlorhexidine will be reduced when mixed with calcium hydroxide but the antibacterial properties of those materials are not loss. These findings indicate that the use of chlorhexidine and calcium hydroxide separately is better to improve antibacterial activity in endodontic treatments. Rahimi *et al* stated that the use of root canal treatment of chlorhexidine as irrigation and calcium hydroxide as medication improved the antibacterial efficacy. The alkaline environment of calcium hydroxide changes the biological properties of lipopolysaccharide (LPS) presented in *F. nucleatum* cell wall, inactivates transport membrane system and kills microorganisms [48]. Previous studies have been reported that *E. faecalis* was resistant to calcium hydroxide effect and mostly found in root canal treatment failure [49]. This type of bacteria has ability to penetrate to dentin and has good adaptation in the environment changes . Chlorhexidine is shown to be effective against some calcium hydroxide strains, so the combination of these medication used separately is considered to have sinergistic activity to increase antibacterial effect [50]. Gomes *et al* evaluated antimicrobial effect of intracanal medications and found that 2% chlorhexidine gel produced the largest zone of inhibition, followed by the combination of calcium hydroxide and iodoform. Chlorhexidine adsorbs on to dentinal tubules and mucous membrane resulting in prolong the release at therapeutics level [51]. Dumani *et al* reported that intracanal medication of calcium hydroxide paste, followed by chlorhexidine solution irrigation worked efficiently in removing *E. faecalis* [52].

5. Conclusions

From this study, it can be concluded that the use of glycerine solvent in calcium hydroxide increases antibacterial activity against *F. nucleatum* and *E. faecalis*. However, the combination is considered because some medicinal materials have their own advantages and disadvantages. The used of various antibacterial agents separately is assumed preferable to avoid antagonistic effects of medications that cause the decrease of antibacterial activities. Further study is needed to obtain proper formulation of

medications to increase antimicrobial and prevent bacterial recolonization and to find successfull root canal therapy.

6. References

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