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Original article

In Vitro Antimicrobial Effectiveness of Calcium Hydroxide and Its Combination with Sodium Hypochlorite against Enterococcus Faecalis and Candida Albicans

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ABSTRACT

The aim of this in vitro study was to evaluate and compare the antimicrobial effect of calcium hydroxide ($Ca(OH)_2$) and its combination with 5% sodium hypochlorite (NaOCL) as intracanal medicaments against Enterococcus faecalis (E. faecalis) and Candida albicans after one, three and seven days. Inoculate of these organisms (E. faecalis and candida albicans) were used to make lawn cultures on Mueller-Hinton with 5% defibrinated sheep blood agar and Mueller-Hinton agar. 60 wells with a depth of 4 mm were prepared for E. faecalis. Then 20 wells filled with $Ca(OH)_2$, 20 wells filled with combination of $Ca(OH)_2$ and 5% NaOCL, and last 20 wells were filled with distilled water as control. A similar procedure was conducted for Candida albicans. The zone of inhibition for each material used against a particular organism were measured and recorded after 1, 3, and 7 days. $Ca(OH)_2$ in combination with 5% NaOCL is more effective than $Ca(OH)_2$ alone against E. faecalis and Candida albicans at different time periods. E. faecalis was more resistant than Candida albicans to intracanal medicaments used. The antimicrobial effect depends on how long it remains inside the root canal. Mixing of $Ca(OH)_2$ with 5% NaOCL had antimicrobial effect on both E. faecalis and Candida albicans and it was more effective against Candida albicans.

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INTRODUCTION

Microorganisms are the cause of apical inflammatory lesions, and the most significant goal of an endodontic treatment is the maximum reduction or elimination of microorganisms and their products from the root canal system. It is difficult to eliminate all microorganism from an infected root canal system by mechanical instrumentation alone [1]. The known recovery of E. faecalis and Candida albicans from failed root canals of teeth in which previous treatment has failed is notable [2,3]. Therefore, chemical irrigation and disinfection are necessary to remove microorganisms, their byproducts and debris from root canal. Intracanal medicaments may perform these roles by remaining in the root canal during treatment appointments [4].

$Ca(OH)_2$ is widely used as an intracanal medication due to its properties such as its tissue dissolving capability, antimicrobial effects, biocompatibility, maintenance in root canal for long time and continuous release of OH-ions [4-7]. However, it has a limited action against some microorganisms, particularly E. faecalis [8] and Candida albicans [9] and requires 60 days to have antimicrobial effect on Candida albicans and E. faecalis [10]. Some studies have reported

the failure of calcium effectively as they tolerate high values of pH [11,12]. In vitro studies have shown that when in direct contact, $Ca(OH)_2$ eliminated microorganisms present in the root canals [13].
NaOCL is currently the most commonly used irrigant with excellent tissue dissolving and antibacterial activities [14,15,16,17]. The antimicrobial effectiveness of NaOCL, based in its high pH (hydroxyl ion action). It interferes with the cytoplasmic membrane integrity with an irreversible enzymatic inhibition, biosynthetic alterations in cellular metabolism and phospholipid degradation. NaOCL can dissolve remnant debris in canal, a property which is desired from an intracanal medicament [18]. Valera et al., [19] stated that 1% NaOCL was an effective in immediate reduction of Candida albicans and E. faecalis counts after root canal preparation. Other studies have confirmed the advantage of NaOCL with other materials. It has been found that the association of NaOCL with $Ca(OH)_2$ shows equal antibacterial activity to $Ca(OH)_2$ and chlorhexidine combination. Zehnder et al., [20] revealed that a quicker antimicrobial activity for$Ca(OH)_2$ with NaOCL in comparison to $Ca(OH)_2$ with water. Farhad et al., [21] stated that the antibacterial activity of $Ca(OH)_2$ and NaOCL did not differ significantly from $Ca(OH)_2$ and chlorhexidine or $Ca(OH)_2$ and water. Suhad et al [22] reported that adding of NaOCL enhance the bactericidal of $Ca(OH)_2$ against microorganisms. In contrast Verrisimo et al [23] concluded that NaOCL showed the worst performance when used alone as intracanal medicament. This is probably occurred because NaOCL losses its antimicrobial properties and becomes ineffective inside the canal after short time. This study was to evaluate and compare the antimicrobial effect of calcium hydroxide ($Ca(OH)_2$) and its combination with 5% sodium hypochlorite (NaOCL) as intracanal medicaments against Enterococcus faecalis (E. faecalis) and Candida albicans after one, three and seven days.

METHODS

In this study, $Ca(OH)_2$ (i-dental, Lithuania), 5% NaOCL (DR. DEPPE, Germany), and microorganisms (E. faecalis and Candida albicans) were used. Agar plates were prepared and used for microorganisms. Aseptic culture was sub-cultured in sterile nutrient broth and incubated at 37ºC for 24 hours. The inoculate of the strains of E. faecalis and Candida albicans were prepared with distilled water. This inoculum was used to prepare a lawn culture of the organisms using sterile cotton swabs on Mueller-Hinton Agar with 5% defibrinated sheep blood.
A total of 15 plates of Muller-Hinton Agar with 5% defibrinated sheep blood agar for E. faecalis were made. Then 4 wells for each plate of diameter 4 mm and depth 4 mm were punched with a sterile punch (60 wells inside 15 plates). The first 20 wells were filled with $Ca(OH)_2$ paste prepared with distilled water (1g $Ca(OH)_2$ powder mixed with 1 ml of distilled water), and the second 20 wells were filled with a combination of 5% NaOCL and $Ca(OH)_2$ paste, and the last 20 wells were filled with distilled water as control. All materials were prepared and used according to the manufacturer's instructions
A total of 15 plates of Mueller-Hinton Agar for Candida albicans were made. A similar procedure was conducted to prepare and fill the 60 wells for Candida albicans (20 wells filled with $Ca(OH)_2$ prepared with distilled water, 20 wells filled with a combination of 5% NaOCL, and $Ca(OH)_2$ and 20 wells filled with distilled water as control).
All the plates were incubated overnight at 37ºC. The specimens were examined after 24 hours, 72 hours and 7 days respectively. The growth inhibition zones around each intracanal medicament were evidenced by the lack of microbial colonization (cleaning of the agar) around each agar well. The zones of inhibition were measured with a transparent ruler, and the 4 mm diameter of the wells was included in the measurement. The wider diameter of the inhibition zone was indicated the higher antimicrobial activity of the intracanal medicament.
The collected data was statistically analyzed by using ANOVA, and the t test. The level of significance was chosen at P=0.05. All statistical analyses were carried out with the SPSS 25 software system.

RESULTS

The mean and standard deviation values for the antimicrobial activity (inhibition zone in mm) of $Ca(OH)_2$ and its combination with 5% NaOCL against E. faecalis and Candida albicans are presented in Table 1 and Figure 1.
The antimicrobial activity of $Ca(OH)_2$ against E. faecalis increased by time, and the zones of microbial growth inhibition (in mm) obtained after one day were 18 ± 0.9176, increased after three days at 20.2 ± 1.1516, and reached their maximum after seven days at 21.75 ± 1.2513.
The antimicrobial activity of $Ca(OH)_2$ and 5% NaOCL combination against E. faecalis increased with time, and the inhibition zone diameters obtained after one day were 19 ± 0.9176 and increased after three days to 21.4 ± 1.6026 and reached to maximum after seven days were 23.4 ± 1.6026
The antimicrobial efficacy of $Ca(OH)_2$ against Candida albicans increased with time, and the diameters of the zones of microbial growth inhibition (in mm) obtained after one day were 20.05 ± 0.8870 and increased after three days to 27.2 ± 1.5761 and reached their maximum after seven days were 30.2 ± 0.8335.

The antimicrobial efficacy of $Ca(OH)_2$ and NaOCL combinations against Candida albicans increased by time, and the diameters of the zones of microbial growth inhibition obtained after one day were 32.85 ± 2.0589, increased after three days to 35 ± 1.6543, and reached of their maximum after seven days were 36.8 ± 2.0416. No antimicrobial activity was observed in the control against for both microorganisms at all time intervals. The adding of 5% NaOCL to $Ca(OH)_2$ recorded higher antimicrobial mean values (inhibition zone) than $Ca(OH)_2$ alone against E. faecalis in all three different investigated periods (24 hours, 72 hours, and 7 days). The inhibition zone values increased with time.

There was a statistically significant difference between the inhibition zones of $Ca(OH)_2$ alone and its combination with 5% NaOCL for E. faecalis after 24 hours, 72 hours, and 7 days ($P < 0.05$). That is, in all of them, equal to ($P = 0.01$). There was a statistically significant difference between the inhibition zones of all tested materials against E. faecalis at all three periods of time ($P < 0.05$) which is equal to ($P = 0.000$). likewise, there was also a statistically significant difference between the control group and each tested material against E. faecalis overall time periods ($P < 0.05$), which is equal to ($P = 0.000$).

The antimicrobial activity of 5% NaOCL combined with $Ca(OH)_2$ exhibited the higher inhibition zone against Candida albicans than $Ca(OH)_2$ after 24 hours, 72 hours, and 7 days with significant statistical difference ($P < 0.05$). That is, in all of them, equal to ($P = 0.000$). All tested materials showed significant statistical difference in the antimicrobial activities against Candida albicans in three different time periods ($P < 0.05$), with P-value of 0.000. The control group revealed significant statistical difference with each tested material in the antimicrobial activity against Candida albicans over all the tested time periods ($P < 0.05$), with a P-value of 0.000. All tested materials $Ca(OH)_2$ and its combination with 5% NaOCL were more effective as antimicrobial against Candida albicans than E. faecalis at 24 hours, 72 hours, and 7 days. In other words, all tested materials have superior antifungal than antibacterial effects over all three periods of time. A statistical analysis showed significant differences in all tested materials between Candida albicans and E. faecalis at all periods of time ($P < 0.05$), which is equal to ($P = 0.000$).

Table 1. Mean values and standard deviation (SD) of the antimicrobial efficacy of $Ca(OH)_2$ and its combination with NaOCL against E. faecalis and Candida albicans

Tested Materials	E. faecalis				Candida albicans			
	Days	NO.	Mean	SD	Days	NO.	Mean	SD
$Ca(OH)_2$	1	20	18	0.9176	1	20	20.05	0.8870
	3	20	20.2	1.1516	3	20	27.2	1.5761
	7	20	21.75	1.2513	7	20	30.2	0.8335
$Ca(OH)_2$ combined with NaOCL	1	20	19	0.9176	1	20	32.85	2.0589
	3	20	21.4	1.6026	3	20	35	1.6543
	7	20	23.4	1.6026	7	20	36.8	2.0416

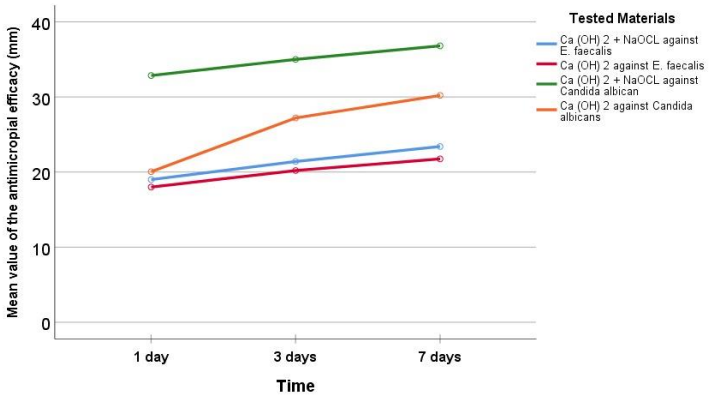

Figure 1. Mean values of the antimicrobial efficacy between E. faecalis and Candida albicans of tested materials after 1 day, 3 days, and 7 days.

DISCUSSION

Radical elimination of microorganisms from infected root canal is difficult task and various measures have been recommended to reduce the numbers of endodontic microorganisms, including the use of intracanal medicaments and irrigation regimens [24].

$Ca(OH)_2$ has been established as antimicrobial agent and it was reported that it may be the best available inter appointment medication [25]. Its antimicrobial activity is related to the release of hydroxyl ions with consequent pH

increase and the inactivation of enzymes of the microorganisms [26]. NaOCL is antimicrobial agent frequently used in root canal therapy as irrigant as well as intracanal medicament [20]. It was reported that hypochlorite acid is formed in the presence water containing active chlorine, a powerful oxidizing agent that produces an antimicrobial effect by disturbing the metabolic functions of microorganism cells [15]. These materials were investigated in this study.

E. faecalis and Candida albicans have been repeatedly identified as the species most commonly recovered from root canals undergoing retreatment in cases of failed endodontic therapy and canals with persistent infections [27,28]. These microorganisms were investigated in this study. The present study resulted that $Ca(OH)_2$ had antimicrobial activity against E. faecalis as demonstrated by the formation of growth inhibition zones. The explanation of this could be related to the lethal effects of hydroxyl ions by unabling the bacteria to survive in the highly alkaline environment [29].

Results of the present study are in line with the findings of Delgado et al., [30] who found that $Ca(OH)_2$ has antimicrobial effects on E. faecalis and Mehrvarzfar et al., [31] who reported that $Ca(OH)_2$ exhibited antimicrobial effects against E. faecalis after 24-, 48-, and 72-hours intervals. On the other hand, the results are in disagreement with the previous studies who concluded that $Ca(OH)_2$ has limited antimicrobial action against E. faecalis [8] and requires 60 days to have antimicrobial effect against E. faecalis [10]. These inconsistencies might be due to the difference in particle proportion and percentage, the type of $Ca(OH)_2$ tested, and the antimicrobial test used [31].

In the current study, the results of combination of $Ca(OH)_2$ with NaOCL revealed high antimicrobial activity against E. faecalis. It also showed that the antimicrobial activity of its combination is greater than $Ca(OH)_2$ by itself with a significant effect on E. faecalis. The antimicrobial activity of NaOCL based on its high pH (hydroxyl ion action). It also interferes with the cytoplasmic membrane integrity with an irreversible enzymatic inhibition, biosynthetic alterations in cellular metabolism and phospholipid degradation [18]. Several studies have confirmed the advantage of combination of $Ca(OH)_2$ with NaOCL. Zehnder et al., [20] reported a quicker antimicrobial effect for $Ca(OH)_2$ mixed with NaOCL in comparison to $Ca(OH)_2$ with water. Suhad et al., [22] concluded that when $Ca(OH)_2$ is mixed with NaOCL, the antimicrobial efficacy of the mixture is greater than $Ca(OH)_2$ by itself. Farhad et al., [21] found that high antimicrobial effectiveness of $Ca(OH)_2$ in combination with NaOCL against E. faecalis. Valera et al., [19] revealed that effectiveness of NaOCL against E. faecalis were noticed. These results are in line with our study.

The present study revealed that $Ca(OH)_2$ showed high antimicrobial activity against Candida albicans. The explanation of this could be related to the action of hydroxyl ions on the inactivation of enzymes of the cytoplasmic membrane of microorganisms and causing toxic effects to their cells [26]. The present results were in agreement with the findings of Valera et al., [19] and McHugh et al., [32] who reported that $Ca(OH)_2$ minimize the growth of Candida albicans. On the other hand, our findings were disagreed with finding of Attia et al., [33]. They observed that $Ca(OH)_2$ had no antifungal activity, so ineffective in eliminating Candida albicans. $Ca(OH)_2$ has a limited antimicrobial action against Candida albicans [9]. Furthermore, Estrela et al., [10] finding did not in consistent with our result, when they concluded that $Ca(OH)_2$ requires 60 days to have an antimicrobial effect on Candida albicans. The explanation for this could be related to testing methodology and materials properties (Attia et al., [33].

The current study also showed stronger antimicrobial effect for $Ca(OH)_2$ with NaOCL in comparison to $Ca(OH)_2$ mixed water. The explanation of this could be attributed to the formation of hypochlorite acid which containing active chlorine and a powerful oxidizing agent that produces an antimicrobial effect by irreversible oxidation of hydrosulphuric group of essential enzymes disturbing the metabolic function of microorganism cell [15]. The results of present study were in line with previous studies done by Hulsmann et al., [34] and Valera et al., [19] who found that NaOCL has been proven to be an effective in eliminating Candida albicans. However, Happasalo & shen [35] and Abbaszadegan et al., [36] reported that NaOCL has limited effect in eliminating microorganisms, which are in contrary with our study. This result may be attributed to the different Candida strains, materials properties and antimicrobial test method.

The present results revealed that all the tested materials ($Ca(OH)_2$ and its combination with NaOCL) record higher antimicrobial activity against Candida albicans than E. faecalis at all periods of time (1,3, and 7 days). This result could be attributed to the fact that E. faecalis was more resistant to high pH environments than another microbe. It can withstand abroad pH range of up to 11.5 and continue to exist after root canal treatment [37]. It is also the bacterium might have other defense mechanisms to withstand this condition and prevent pH damage [38].

CONCLUSION

Both $Ca(OH)_2$ and its combination with 5% NaOCL showed antimicrobial and antifungal effect. E. faecalis was more resistant than Candida albicans to both tested groups. The combination of $Ca(OH)_2$ with 5% NaOCL has higher antimicrobial activity than $Ca(OH)_2$ mixed with water against both E. faecalis and Candida albicans. The antimicrobial property was increased over time for all tested materials. The antimicrobial effect depends on how long it remains inside the root canal.

Conflicts of Interest

The authors declare no conflicts of interest.

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دراسة معملية بخصوص الفعالية المضادة للميكروبات لهيدروكسيد الكالسيوم وتوليفته مع هيبوكلوريت الصوديوم ضد المكورات المعوية البرازية والمبيضات المبيضة

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المستخلص

كان الهدف من هذه الدراسة المعملية هو تقييم ومقارنة التأثير المضاد للميكروبات لهيدروكسيد الكالسيوم وتوليفته مع هيبوكلوريت الصوديوم 5% كأدوية داخل القناة ضد المكورات المعوية البرازية و المبيضات المبيضة بعد يوم وثلاثة وسبعة أيام . تم استخدام التلقيح من هذه الكائنات الحية (المكورات المعوية البرازية و المبيضات المبيضة) في زراعة العشب في مولر-هينتون باستخدام أجار دم الأغنام منزوع الرجفان بنسبة 5% وأجار مولر-هينتون. تم تحضير 60 بئراً بعمق 4 ملم للمكورات المعوية البرازية . ثم تم ملء 20 بئرًا بهيدروكسيد الكالسيوم، و20 بئرًا مملوءة بمزيج من هيدروكسيد الكالسيوم و5% هيبوكلوريت الصوديوم ، وتم ملء آخر 20 بئرًا بالماء المقطر كعنصر تحكم. تم إجراء إجراء مماثل للمبيضات المبيضة. تم قياس وتسجيل منطقة التثبيط لكل مادة مستخدمة ضد كائن حي معين بعد 1، 3، و7 أيام . يعتبر هيدروكسيد الكالسيوم مع 5% هيبوكلوريت الصوديوم أكثر فعالية من هيدروكسيد الكالسيوم وحده ضد المكورات المعوية البرازية والمبيضات المبيضة في فترات زمنية مختلفة. كانت المكورات المعوية البرازية أكثر مقاومة من المبيضات المبيضة للأدوية المستخدمة داخل القناة. يعتمد التأثير المضاد للميكروبات على المدة التي تبقى فيها داخل قناة الجذر. كان لخلط هيدروكسيد الكالسيوم مع 5% هيبوكلوريت الصوديوم تأثير مضاد للميكروبات على كل من المكورات المعوية البرازية والمبيضات المبيضة وكان أكثر فعالية ضد المبيضات المبيضة .

الكلمات الدّالة. هيدروكسيد الكالسيوم، هيبوكلوريت الصوديوم، المكورات المعوية البرازية، المبيضات المبيضة.