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Antibacterial Activity of Calcium Hydroxide Combined with Chlrohexidine or Sodium Hypochlorite against Gram Positive and Gram Negative Bacteria

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Abstract
The most significant current goal in endodontology is the maximum reduction of the microorganisms because they play a fundamental role in the etiology of pulpo-periapical pathosis and may escape from tooth and circulate throughout the body to cause an infection in distant part. Numerous materials have been used to achieve this goal. Calcium hydroxide [Ca(OH)2]with its unique properties, need to be mixed with other agents to fulfill its antimicrobial requirement. However, to the best of authors knowledge , no report has been found so far investigates the antimicrobial efficacy of Calcium hydroxide / chlorhexidine [CHX]combination against klebsiella spp..The aim of this study is to evaluation of the effect of adding CHX or sodium hypochlorite[NaOCl] on the antibacterial activity of Ca(OH)2 against gram positive and gram negative bacteria. The tested samples were grouped into 5 groups of 20 samples for each group: G1:Ca(OH)2 mixed with H2O. G2:2% CHX solution. G3: 2.5% NaOCl solution. G4: combination of Ca(OH)2 and 2% CHX. G5:combination of Ca(OH)2 and 2.5% NaOCl. 10 samples from each group had been inoculated with streptococcus spp. and the other 10 had been inoculated with klebsiella spp. Microbial growth was verified and number of plates which show growth or no growth for bacteria were counted and analyzed statistically. As a result Ca(OH)2 and 2.5% NaOCl had antibacterial effect on both tested bacteria while 2% CHX appeared to be less effective . Pastes of Ca(OH)2 and 2% CHX were effective on klebsiella spp., while mixing of Ca(OH)2 with 2.5% NaOCl had significant effect on both types of bacteria and it was more effective against streptococcus spp.
Key words: Calcium hydroxide, Chlorhexidine, Sodium hypochlorite, klebsiella, Streptococcus .

1. Introduction

Bacteria play a fundamental role in the etiology of pulpo-periapical pathosis (Haapasalo & Qian 2008)and non vital teeth have been considered as foci of infection . They are sources for bacteria with their toxins which may escape from the tooth and circulate throughout the body to localize on a new tissue and cause an infection in distant part of body (Murray & Saunders 2000& Walsh1997).The polymicrobial nature of endodontic infection has been stated, where predominant microbial groups frequently isolated from infected root canal are the aerobic and facultative anaerobic organisms (Baumgartner &Falker 1991). Among these streptococcus spp. are one of the most commonly identified bacteria penetrating the root dentinal tubules and considered as a major cause of endodontic failure (Perez et.al 1993& Gajan et al. 2009). It was established that streptococci, enterococci and lactobacilli appear to commonly survive and recover following root-canal treatment of non-vital teeth with apical periodontitis (Chávez et al.2003). Besides that , the transformation of streptococcus to enterocuccus faecalis had been reported (Schleifer & Kilpper-Balz 1984). Regarding the gram negative bacteria ,it was also reported that klepsiella has been isolated from non vital teeth (Little 1975) and may play a significant role in clinical condition of flaring up where it was determined as a cause (Chaudhry et al.1997). Klebsiella spp. frequently causes pneumonia, urinary tract infection, wound infection, and bacteremia (Podschun & Ullmann 1998). Klebsiella has developed resistant to chlorhexidine which could be clinically more problematic (Hammond et al. 1987&Thomas et al. 2000).

Maximum reduction of the root canal microorganisms has been introduced as a major goal of endodontic therapy. It may be achieved with the aids of numerous materials (Bystrom & Sundqvist 1981). Ca(OH)2 has unique properties to be considered as an ideal root canal dressing such as tissue dissolving capability, antimicrobial effect, biocompatibility and maintenance in root canal for a long time. It also promotes an alkalinizing osteogenesis through the continuous release of OH- ions (Mustafa et al.2012). However, Ca(OH)2 cannot be considered as a universal intracanal medicament, since it is not equally effective against all bacteria found in the root canal. In fact, several studies have reported the failure of Ca(OH)2 to eliminate enterococcus faecalis effectively as they tolerate high pH values (Gomes et al.2002&Pinheiro et al.2003). Ca(OH)2 mixed with CHX to fulfill antimicrobial requirements of an intracanal medicament (Valera et al.2009&Farhad et al.2012).

CHX has been used as an irrigant as well as an intracanal medicament. The antimicrobial effect of CHX is related to the cationic molecule binding to negatively charged bacterial cell walls, thereby altering the cell's osmotic equilibrium (Mohammadi & Abbott 2009). Many researchers reported that CHX was the more effective

than NaOCl against anaerobic bacteria (Ohara et al.1993& Jeansonne & White 1994). Ferraz et al.(2001) also claimed the antimicrobial property of 2% CHX gel. When used as an intracanal medicament, CHX was more effective than Ca(OH)2 against enterococcus faecalis infection in dentinal tubules. Gram-negative bacteria are less susceptible to CHX than gram-positive bacteria (Gomes et al. 2001& Hugo 1992). Many types of bacteria may develop resistance to CHX, including klebsiella (McDonnell & Russell AD1999).

NaOCl has been represented as the most popular irrigant with excellent tissue dissolving and antibacterial activities (Thomas et al.2000). Beside their wide spectrum with non-specific killing effects on all microbes, hypochlorite preparations are sporicidal, viricidal (Weine 1982), and show far greater tissue dissolving effects on necrotic than on vital tissues (Austin & Taylor 1918). 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 (Estrela et al.2002). Valera et al.(2009) stated that 1% NaOCl was effective in immediate reduction of candida albicans and enterococcus faecalis counts after root canal preparation. In contrast Verrisimo et al.(2010) reported that NaOCl showed the worst performance when used alone as intracanal medicament. That this probably occurred because NaOCl loses its antimicrobial properties and becomes ineffective inside the canal after short period of time.

More recent studies have confirmed the advantage of combination of Ca(OH)2 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 CHX combination. Zehnder et al.(2003) reported a quicker antimicrobial effect for Ca(OH)2 with NaOCl in comparison to Ca(OH)2 with water. Farhad et al.(2012) stated that using of CHX as a vehicle to Ca(OH)2 revealed more significant antibacterial effect than its mixture with H2O. The antibacterial activity of Ca(OH)2 and NaOCl did not differ significantly from Ca(OH)2 and CHX or Ca(OH)2 /H2O mixtures. Unlike NaOCl , CHX has a property the substantivity which allows prevention of microbial colonization on dentine surface for some time beyond the actual period of time of medicament application (Athanassiadis et al.2007). On the other hand ,NaOCl can dissolve remnant debris in canal, a property which is desired from an intracanal medicament. However, NaOCl has limited capacity to penetrate into dentinal tubules (Valera et al. 2009).

The aim of this study is to evaluate the effect of adding CHX or NaOCl on the antibacterial activity of Ca(OH)2 against gram positive and gram negative bacteria which had been isolated from primarily infected root canals.

2. Materials and Methods

2.1 Sample selection

This study was carried out in the dental clinics of College of Dentistry in University of Kufa during the period between September to December 2013. The bacterial samples were randomly selected from 40 patients. Each patient have at least one symptomatic non vital tooth of an X-ray of periapical involvement.

2.2 Collection of specimens

The bacterial sample were taken under aseptic conditions. The involved tooth was isolated. The field was disinfected and the accessed cavity was prepared with a sterile round bur. After gaining access to the pulp, a sterile reamer was inserted apically into the root and root canal content were obtained for culture. Reamer containing root canal contents was placed in tubes containing brain heart infusion broth. Then all the tubes immediately transferred to the laboratory for bacteriological study (McFadden 2000).

2.3 Isolation of bacteria:

Root canal contents were inoculated on blood agar and MaConkey agar then incubated at 37oC for 24 hours. Colony characteristics were noted in case of any growth and identification of microorganisms was done according to the morphology using gram staining and by biochemical reactions (Oguntebi et al.1982).

Culturing: the isolated bacteria test : streptococcus spp. and klebsiella spp. were subcultured onto appropriate culture media under gaseous conditions for 48 hours on sheep blood-brain heart infusion (BHI) agar plates at 37 oC for 24 hours.

The tested materials were grouped into 5 groups of 20 samples for each group:

G1: sterile paste made of calcium hydroxide mixed with water at 1mg/1ml (Ca(OH)2/H2O)

G2: 2% chlorhexidine solution (CHX).

G3: 2.5% sodium hypochlorite solution (NaOCl).

G4: sterile paste made of calcium hydroxide mixed with 2% chlorhexidine at 1g/1ml (Ca(OH)2/CHX).

G5: sterile paste made of calcium hydroxide mixed with 2.5% sodium hypochlorite at 1g/1ml (Ca(OH)2/NaOCl).

10 samples from each group had been inoculated with streptococcus spp. and the other 10 had been inoculated with klebsiella spp. Then all groups cultured under gaseous conditions on sheep blood-brain heart infusion (BHI) agar plates at 37 oC for 24 hours. With respect to the 24 hours bacterial growth plates which showed no growth were further incubated up to 24 hours before deciding it as negative result. Microbial growth was verified by gram stain and light microscope.

2.4 Statistical analysis :

Microbial growth was verified and number of plates which show growth or no growth for streptococcus spp. or klebsiella spp. were counted and analyzed statistically using Chi-square with p-value 0.05.

3-Result

Table (1):Growth comparison of streptococcus and klebsiella in relation to tested materials

Tested materials	Streptococcus spp.		Klebsiella spp.		Chi square	p- value
	No.	Growth	No.	Growth		
Ca(OH)2	10	6	10	4	0.8	0.3
CHX	10	8	10	9	0.392	0.53
NaOCl	10	5	10	6	0.202	0.65
Ca(OH)2/CHX	10	4	10	2	0.95	0.32
Ca(OH)2/NaOCl	10	2	10	8	7.2	0.007

Table (1) illustrates the number of plates of streptococcus ssp. and klebsiella spp. which shows growth or no growth after treatment with tested materials .Paste of Ca(OH)2 /H2O had an effect against both types of bacteria with no significant statistical difference .

2% CHX appeared to be less effective with no significant statistical difference in the antibacterial effectiveness of 2% CHX against both types of bacteria.

2.5%NaOCl revealed to be effective against both types of bacteria with no significant statistical difference in the antibacterial effectiveness of 2.5%NaOCl on streptococcus spp. or klebsiella spp..

The more surprising correlation is with the adding of 2% CHX to Ca(OH)2 had synergistic effect on its antibacterial activity against both types of bacteria with significant effect on klebsiella spp. While mixing of NaOCl with Ca(OH)2 affected both types with higher effect on streptococcus spp..

Table (2): Comparison of Ca(OH)2 with Ca(OH)2/CHX

Bacterial isolate	Ca(OH)2 /H2O		Ca(OH)2 /CHX		p- value
	Growth	No growth	Growth	No growth	Chi square =1.752
Streptococcus spp	6	4	4	6	p- value = 0.62
Klebsiella spp	4	6	2	8	
Total	10	10	6	14	

As shown in table(2), There was no statistical significant differences between antibacterial activity of Ca(OH)2 and Ca(OH)2/CHX .

Table(3):Comparison of Ca(OH)2/H2O with Ca(OH)2/NaOCl

Bacterial isolate	CaOH2/H2O		CaOH2/ NaOCl		p- value
	Growth	No growth	Growth	No growth	Chi square =8.0
Streptococcus spp.	6	4	2	8	p- value =0.04
Klebsiella spp.	4	6	8	2	
Total	10	10	10	10	

Table(3): reveals a comparison between the antibacterial activity of Ca(OH)2/H2O and Ca(OH)2/NaOCl . There are significant statistical differences with encouraging effect especially on streptococcus spp.

Table(4):Comparison of Ca(OH)2/H2O with Ca(OH)2/NaOCl

Bacterial isolate	Ca(OH)2 /CHX		Ca(OH)2 /NaOCl		p- value
	Growth	Growth	No growth	No growth	Chi square =8.15
Streptococcus spp.	2	4	6	8	p- value = 0.042
Klebsiella spp.	8	2	8	2	
Total	10	6	14	10	

According to table (4) ,there was significant difference between the Ca(OH)2/CHX and Ca(OH)2/NaOCl.

Table(5):Comparison of 2% CHX with Ca(OH)2/CHX on streptococcus spp.

Bacterial isolate	CHX		Ca(OH)2 /CHX		p- value
	Growth	No growth	Growth	No growth	Chi square = 4
Streptococcus ssp	8	2	4	6	p- value = 0.261
Total	10		10		

Table(5) compares the antibacterial activity of 2% CHX and Ca(OH)2/CHX on streptococcus spp. There was no significant statistical difference between two tested materials.

Table(6): Comparison of 2% CHX with Ca(OH)2/CHX on klebsiella spp.

Bacterial isolate	2%CHX		Ca(OH)2 / CHX		p- value
	Growth	No growth	Growth	No growth	Chi square = 10
Klebsiella ssp.	9	1	2	8	p- value = 0.018
Total	10		10		

Table(6) evaluates the antibacterial activity of 2%CHX and Ca(OH)2+CHX on Klepsiella spp. .There was significant statistical synergistic effect.

Table(7):Comparison of 2.5% NaOCl with Ca(OH)2/NaOCl

Bacterial isolate	2.5%NaOCl		Ca(OH)2 /NaOCl		p- value
	Growth	No growth	Growth	No growth	Chi square =7.40
Streptococcus ssp.	5	5	2	8	p- value =0.06
Klebsiella ssp.	6	4	8	2	
Total	10	10	10	10	

Table (7) compares the antibacterial activity of2%NaOCl and Ca(OH)2/NaOCl on both types of bacteria . There was significant statistical difference with an interesting effect for Ca(OH)2/NaOCl on G+ve bacteria.

4. Discussion

Radical elimination of microorganisms from infected root canals is a difficult task and various measures have been recommended to reduce the numbers of endodontic microorganisms, including the use of irrigation regimens and intracanal medicaments (Gomes et al.1996). Ca(OH)2 has been established as antimicrobial agent and it was reported that it may be the best available inter appointment medication (Law & Messer 2004). The present study revealed its effectiveness against the streptococcus and klebsiella spp.. That is in agreement with the finding of Gomes et al. as they stated that, Ca(OH)2 is equally effective against all bacteria found in the root canal (Gomes et al.2002) .

Its high pH (approximately 12-12.5) has a destructive effect on bacterial cell membranes and protein structures (Mustafa et al.2012). However, Ca(OH)2 failed to eradicate all the tested bacteria. It has been suggested to mix Ca(OH)2 powder with antimicrobial endodontic irrigants to obtain a wider antimicrobial spectrum with a long lasting effect (Waltimo et al.1999). Different vehicles have been added to Ca(OH)2 in an attempt to improve its antimicrobial activity, biocompatibility, speed of ionic dissociation, and diffusion (Fava & Saunders 1999).

NaOCl and CHX are antimicrobial agents frequently used in root canal therapy as irrigant as well as intracanal medicament (Zehnder et al.2003). Efficacy of CHX is because of interaction of the positive charge of its molecules with the negatively charged phosphate groups on microbial cell walls (Mohammadi & Abbott 2009). CHX has wide spectrum antimicrobial activity and prolonged action. Cervone et al (1990) demonstrated that CHX has inhibitory effects on bacteria commonly found in endodontic infections while other researcher stated that gram-negative bacteria are less susceptible to CHX than gram-positive bacteria (McDonnell & Russell 1999). However , in the present research it appeared to be less effective against both types of bacteria.

The combination of Ca(OH)2 and CHX has been used with encouraging result (Mohammadi &Abbott 2009 &Gomes et al.2006 &Evans et al.2003). The aim of combining Ca(OH)2 and CHX is to increase the antimicrobial properties of Ca(OH)2 and act as an adjunct to destroy bacteria due to its antimicrobial activity and substantivity (Gomes et al.2002). A number of studies using in vitro or in vivo models have stated that the antimicrobial efficacy of CHX/Ca(OH)2 against enterococcus. faecalis is more than Ca(OH)2 alone (Cervone et al.1990 & Ercan et al. 2006) while others using different study designs have not found the same results (Schafer & Bossmann 2005& Zerella et al.2005). We didn't find a research has investigated the antimicrobial effect of Ca(OH)2 /CHX combination against klepseilla spp.

This study illustrated the support of the CHX to the bacteriocidal effect of Ca(OH)2 with interesting effect on klebsiella. Regarding the effect of this mixture against streptococcus , our result was in disagreement with the finding of Manzur et al.(2007) who assessed the antibacterial efficacy of intracanal medication with Ca(OH)2, 2%CHX gel and a combination of both Ca(OH)2 and CHX in teeth with chronic apical periodontitis. They concluded that the antibacterial efficacies of Ca(OH)2, CHX and mixture of Ca(OH)2/CHX were comparable. The finding of this study is comparable with result of Silvera et al.(2011) as they assessed antibacterial activity of four formulations of Ca(OH)2 pastes against Streptococcus as one of tested bacteria .They found that the association of Ca(OH)2 and CHX showed a better performance than Ca(OH)2 alone.

The present study has revealed the effectiveness of NaOCl against both bacteria. The effectiveness of NaOCl as an irrigant solution has been confirmed by several researches . It is widely used in endodontic and 2.5% NaOCl concentration was selected because this is a commonly used in endodontic treatment. It can preserve sufficient amount of chlorine to eliminate significant amount of bacterial , comparable with effect of a higher concentration

(Siquiria et al.2000).
The present study revealed that the NaOCl had stronger antibacterial effect than the CHX. This is supported by finding of Sprat et al.(2001). They studied the effectiveness of 2.25%NaOCl, 0.2%CHX and 10% povidone iodine against five root canal isolated bacteria including P.intermedia, streptococcus intermedius , peptostreptococcusmiros , fusobacteriumnucleatum and enterococcus faecalis . They reported that NaOCl was the most effective antimicrobial agent followed by iodine solution.
On the other hand , our finding was disagree with the finding of Sequeira et al. (2007) .They compared the effectiveness of 2.5%NaOCL and 0.12%CHX. They found that both irrigants had the ability to reduce the cultivable bacteria in infected root canals. Furthermore, Ercan et al.(2004) finding didn't in consistent with our research, when they evaluated the antibacterial efficacy of 2% CHX and 5.25% NaOCl in infected root canals of incisor and premolars They concluded that both CHX and NaOCl were effective irrigants for reducing the number of microorganisms in teeth with necrotic pulp and/or periapical pathosis .
In comparison of Ca(OH)2, CHX and NaOCl ,the present study has illustrated that there was no significant difference between effect of Ca(OH)2 and NaOCl on streptococcus spp. and klebsiella spp. with antibacterial stronger than CHX. This in disagreement with statement of Elca et al. (2005). They conducted study to evaluate their antibacterial effects against streptococcus pyogenes, streptococcus mitis, streptococcus bovis, streptococcus anginosus , enterococcus faecalis, staphylococcus aureus. Ca(OH)2 had the strongest impact in direct contact with the studied microorganisms, followed by 0.1% CHX and 3% NaOCl .
Our research verified that when Ca(OH)2 is mixed with NaOCl , the antimicrobial efficacy of the mixture is greater than Ca(OH)2 by itself with significant effect on streptococcus. To some extent, this finding is comparable to result of Farhad et al.(2012) as they compared the antibacterial effectiveness of Ca(OH)2 in combination with H2O, CHX, or NaOCl against enterococcus faecalis. Their findings indicated that the antibacterial potency of Ca(OH)2 can be enhanced by preparing it with antibacterial irrigants such as CHX or NaOCl .

5.Conclusion

Within the limitation of this experimental study , involving only two bacterial species , it can be concluded that adding of CHX may enhance the antibacterial activity of Ca(OH)2 against klebseilla spp. as gram negative bacteria ,while adding of NaOCl enhance the bactericidal of Ca(OH)2 against both types of bacteria with interesting effect on streptococcus spp. as gram negative bacteria . However ,they were not able to produce complete eradication of bacteria.
Till date there is no literature investigate the antimicrobial efficacy of Ca(OH)2/CHX combination against klebsiella spp..

6. Suggestion

1-Further research is strongly recommended to investigate the antimicrobial effect of the combinations against other types of microorganisms and using different study methodology.
2-Further studies should be done to secure the biocompatibility of Ca(OH)2 combination as an introduction for a clinical application as intracanal medicament , periodontal pack and infected wound dressing.

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