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http://dx.doi.org/10.1590/1678-775720140032

Antibacterial effect of calcium hydroxide combined with chlorhexidine on *Enterococcus faecalis*: a systematic review and meta-analysis

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Submitted: January 24, 2014 - Modification: April 30, 2014 - Accepted: May 15, 2014

ABSTRACT

Objective: *Enterococcus faecalis* (*E. faecalis*) is the most frequently isolated strain in failed endodontic therapy cases since it is resistant to calcium hydroxide (CH). Whether a combination of CH and chlorhexidine (CHX) is more effective than CH alone against E. faecalis is a matter of controversy. Thus, the aim of this study was to conduct a systematic review and meta-analysis of the literature. Material and Methods: A comprehensive search in PubMed, EMbase, EBSCOhost, The Cochrane Library, SciELO, and BBO databases, Clinical trials registers, Open Grey, and conference proceedings from the earliest available date to February 1, 2013 was carried out and the relevant articles were identified by two independent reviewers. Backward and forward search was performed and then inclusion and exclusion criteria were applied. The included studies were divided into "comparisons" according to the depth of sampling and dressing period of each medicament. Meta-analysis was performed using Stata software 10.0. The level of significance was set at 0.05. Results: Eighty-five studies were retrieved from databases and backward/forward searches. Forty-five studies were considered as relevant (5 *in vivo*, 18 *in vitro*, 18 *ex vivo*, and 4 review articles). Nine studies were included for meta-analysis. Inter-observer agreement (Cohen kappa) was 0.93. The included studies were divided into 21 comparisons for meta-analysis. Chi-square test showed the comparisons were heterogeneous ($p<0.001$). Random effect model demonstrated no significant difference between CH/CHX mixture and CH alone in their effect on *E. faecalis* ($p=0.115$). Conclusions: According to the evidence available now, mixing CH with CHX does not significantly increase the antimicrobial activity of CH against *E. faecalis*. It appears that mixing CH with CHX does not improve its *ex vivo* antibacterial property as an intracanal medicament against *E. faecalis*. Further *in vivo* studies are necessary to confirm and correlate the findings of this study with the clinical outcomes.

Keywords: Products with antimicrobial action. Calcium hydroxide. Chlorhexidine. *Enterococcus faecalis*. Meta-analysis.

INTRODUCTION

Microbial invasion of the root canal system has an important role in initiating and sustaining periapical disease[41]. The aim of root canal therapy is to eliminate bacteria and their by-products from the root canal system[74]. Although chemomechanical cleaning and shaping of the canal is effective in reducing bacterial counts, microorganisms may persist in the anatomical complexities of root canal system and increase the risk of treatment failure[12,109]. Therefore, intracanal medication is advocated to further reduce bacteria in the root canal system and increase the success of root canal treatment[11].

Calcium hydroxide (CH) is the most commonly used intracanal medicament in endodontics[93]. It dissociates into calcium and hydroxyl ions in an aqueous solution. The antimicrobial property of CH is attributed to the release of hydroxyl ions and provides a highly alkaline environment with a pH value of approximately 12.5[93,101]. Most of the microorganisms in infected root canals are unable to survive in the alkaline environment[37]. However, CH is not equally effective against all the bacteria found in the root canal[70].

Chlorhexidine gluconate (CHX) can be used in endodontics as an irrigant and intracanal medicament due to its biocompatibility, substantivity and wide antimicrobial activity[17,18]. The antimicrobial property of CHX is attributed to its cationic molecule, which is adsorbed to the negatively charged inner cell membrane, resulting in the leakage of intracellular components. It is an effective agent against gram-positive and gram-negative bacteria[39]. Importantly, it is effective against microorganisms resistant to CH[90].

Enterococcus faecalis (*E. faecalis*) is a gram-positive facultative anaerobic bacteria species. It is one of the most CH-resistant microorganisms of the root canal system[100]. Although it comprises a small proportion of the root canal flora in initial endodontic infections, environmental changes can be advantageous to *E. faecalis*, resulting in persistent infections[99]. Some resistance factors of this bacterial species are deep dentinal penetration ability[33], high pH tolerance[19], surviving in food deprivation condition[100], and surviving without any support from other microbial species[70].

Many studies have attempted to compare antibacterial effect of CH alone or in combination with CHX. Some studies have shown an increased antibacterial effect when CHX is added to CH[8,14,15,20,80], while other studies have failed to show any benefits in incorporating CHX[4,55,85,98]. It seems that the usefulness of mixing CH with CHX remains unclear and controversial[61]. Therefore, the aim of this systematic review and meta-analysis was to determine whether adding CHX to CH can improve the efficacy of CH against *E. faecalis* in dentinal tubules or not.

MATERIAL AND METHODS

Review question

The following well-defined review question was developed by using the Population, Intervention, Comparison, and Outcome (PICO) framework: Does CH/CHX mixture (I), compared to CH alone (C), result in higher antimicrobial efficacy (O) against *E. faecalis* (P) in infected dentin? Therefore, the key words for search strategy were "*Enterococcus faecalis*" and "*E. faecalis*" as Population, "chlorhexidine" as Intervention, "calcium hydroxide" as Comparison, and "antimicrobial" and "antibacterial" as Outcome.

Search strategy

A comprehensive search of the literature was performed in Medline (PubMed), EMbase, EBSCOhost, The Cochrane Library SciELO, and BBO databases from the earliest available date to February 1, 2013 by an expert researcher in health and medical sciences (HN). Also, unpublished data, abstracts, and gray literature were sought through clinical trials registries (Australian New Zealand Clinical Trials Registry, Brazilian Clinical Trials Registry, Iranian Registry of Clinical Trials, United States National Institutes of Health, ClinicalTrials.gov, World Health Organization International Clinical Trials Registry Platform, and European Union EU Clinical Trials Registry), Open Grey, and conference proceedings.

The key words were organized according to the PICO model and were ["*Enterococcus faecalis*" OR "*E. faecalis*"] AND chlorhexidine AND "calcium hydroxide" AND [antimicrobial OR antibacterial]. No limitations were implemented by country of origin, language or date. The identified studies were combined using the bibliographic software EndNote X4 (Thomson Reuters, Carlsbad, CA, USA).

Study selection and data extraction

Two independent reviewers (MS, AS) screened the titles and abstracts of all the identified studies to determine relevant studies which met predetermined inclusion criteria. If there were insufficient data to make a clear decision, the full text was considered. Backward and forward searches from the relevant studies were also conducted, and the references of relevant studies were checked as backward search. Also, a forward search was undertaken on the titles of the relevant studies. Articles that had cited these studies were also identified through http://www.scholar.google.com to identify potentially relevant subsequent primary research.

These two independent reviewers assessed the full texts of relevant studies based on inclusion and exclusion criteria, which were proposed by three professionals related to each part of the study: two endodontists (MS, AS), an epidemiologist (MM), and a microbiologist (HS).

Inclusion criteria were as follows:

In vivo or *ex vivo* study using dentin block model microbiological assessment

Human or bovine dentin

CH in combination with CHX in the CH/CHX group

CH in combination with distilled water or saline in the CH-alone group

E. faecalis as a strain for microbiological assessment

Dressing period of at least 1 day

Quantitative results provided

Exclusion criteria were as follows:

Review article

In vitro study

Vehicles other than distilled water or saline for CH-alone group

CH or CHX as medicament in other materials

Any intervention except medicament dressing for bacterial elimination

Qualitative results or invalid means and standard deviations (SD) reported

Any disagreements on study inclusion and exclusion criteria were discussed and resolved by consulting a third reviewer.

Data extraction, synthesis and analysis

The included studies were reviewed and divided into "comparisons" according to dressing periods and depths of sampling. Sample size, microbiologic unit, depth of sampling, type and concentration of CHX, significance, and means (SD) were recorded for each comparison individually. A microbiological unit for two included studies[8,98] was optical density (OD) and for other included studies it was the colony forming unit (CFU). In order to identify the measurement scale, the results of these two studies were transformed from OD to CFU according to microbiological equation (OD of 0.5 corresponding to $\sim 5\times10^8$CFU/mL)[36]. The results of six included studies[4,8,15,20,55,98] were converted to the logarithm of CFU in order to achieve identical data for meta-analysis. Since SD had not been reported in some studies[15,55,98], it was estimated and used for further analysis by using formula of t-test and application of means, sample size, and p value of each study.

Statistical analysis was performed using Stata software version 10.0 for Windows (Stata Corp LP, College Station, Texas, USA). The level of significance was set at 0.05. After checking the heterogeneity of comparisons using Chi-square analysis, Random-effect meta-analysis model was used to estimate the combined effect. The results of these comparisons were represented by Forest plot. The potential risk of publication bias was evaluated using Begg's and Egger's tests.

Although the antibacterial effect of medicaments was evaluated with different depths of sampling ranges (from 0.05 mm to 0.45 mm) in the included studies, subgroup analysis was performed based on the depth of sampling. Therefore, the comparisons were divided into surface (depth of sampling ≤0.2 mm) and deep (depth of sampling >0.2 mm) dentin groups.

RESULTS

The results of the search strategy are presented in Figure 1. Figure 2 presents a flow chart of the systematic review process. The final results of the search in Medline (via PubMed), EMbase, EBSCOhost, The Cochrane Library, SciELO, and BBO were 77, 65, 17, 3, 4, and 7 studies, respectively. After the primary review, 44 studies[2-8,14-16,20,22,26-30,35,40,50,52,55-57,62-64,67,68,71,72,80,85,89,95,97,98,102,104,105,107,113-115] were considered relevant, and 40 studies[1,9,10,13,19,24,25,31,34,38,42-44,46-49,51,53,54,58-60,65,66,69,75-79,81,83,86,94,103,106,110-112] were irrelevant. Inter-observer agreement (Cohen kappa) was 0.93. One additional study[82] was considered relevant by backward and forward search. Thus, 45 studies were considered as irrelevant (5 *in vivo*, 18 *in vitro*, 18 *ex vivo*, and 4 review articles). By implementation of inclusion and exclusion criteria, nine studies[4,8,14,15,20,55,80,85,98] were included and 36 studies[2,3,5-7,16,22,26-30,35,40,50,52,56,57,62-64,67,68,71,72,82,89,95,97,102,104,105,107,113-115] were excluded (Figure 3).

Twenty-one comparisons from the nine included studies were extracted (Table 1). Eight comparisons showed no significant differences in antibacterial effects between CH and CH/CHX mixture; ten comparisons showed significant differences in favor of CH/CHX mixture; and 3 comparisons showed significant differences in favor of CH alone against *E. faecalis*. The 21 comparisons were heterogeneous (Cochran Q Test of Homogeneity ($\chi2$)=144.22, df=20, p<0.001). Therefore, random effect method for combining comparison estimates was used and an overall estimate was produced. There were no significant differences in antibacterial effects between CH/CHX mixture and CH alone against *E. faecalis* (p=0.115) (Figure 4A).

The estimated ranks of correlation coefficients of Begg's and Egger's tests were 0.21 (p=0.809) and 0.23 (p=0.215), respectively, which means that there is no evidence for considerable publication bias in this study (Figure 4B).

In addition, subgroup analysis showed no

Entry	Results					
	PubMed	EMbase	EBSCO	Chochrane	SciELO	BBO
#1: "*Enterococcus faecalis*" OR "*E. faecalis*"	11298	18879	2938	194	218	197
#2: Chlorhexidine	7664	14568	2167	2222	192	438
#3: "Calcium hydroxide"	4259	4599	1540	296	163	703
#4: antibacterial OR antimicrobial	1286445	2250318	80454	7143	2775	447
#5: #1 AND #2 AND #3 AND #4	77	65	17	3	4	7

Figure 1- Search strategy through PubMed, EMbase, EBSCOhost, The Cochrane Library, SciELO, and BBO

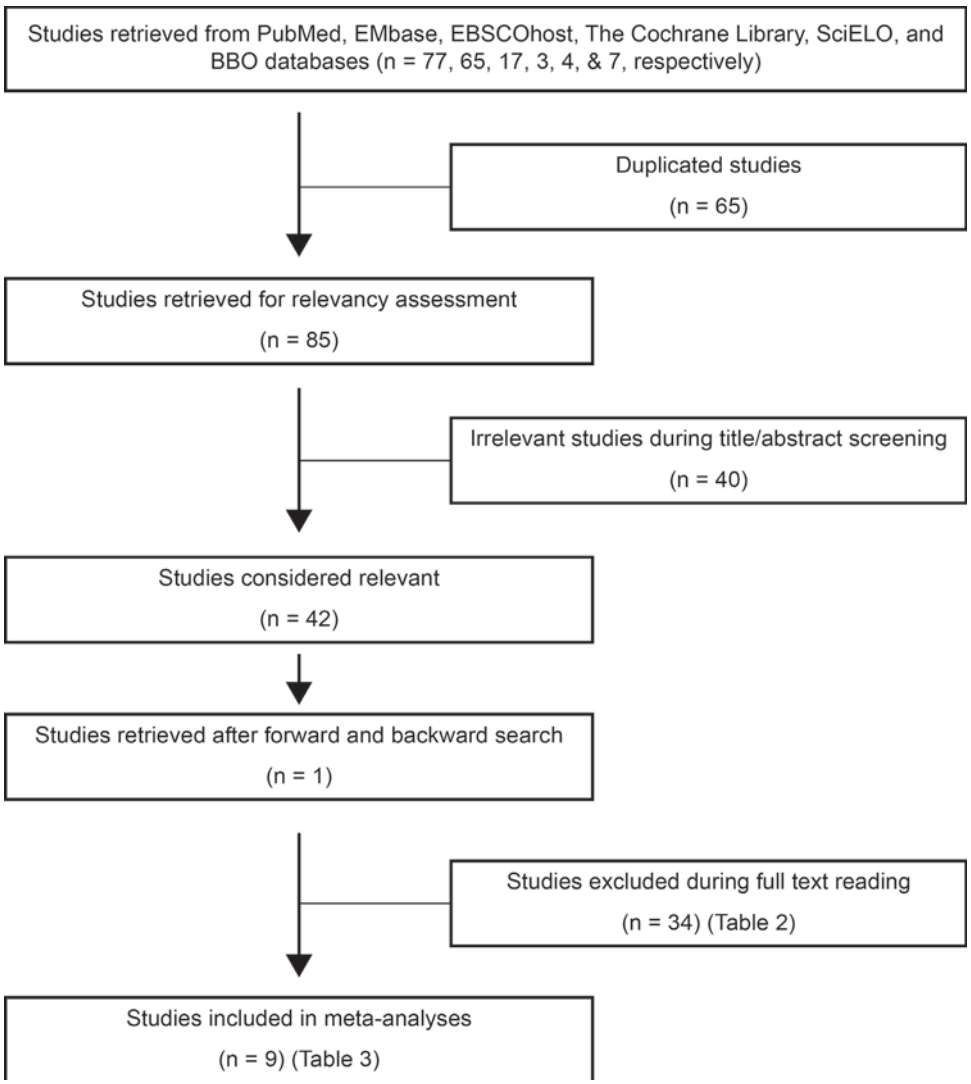

Figure 2- Flow chart of the search strategy

significant differences in antibacterial effects between CH/CHX mixture and CH alone against *E. faecalis* in the surface (p=0.11) and deep (p=0.57) dentin (Figures 5A and 5B).

DISCUSSION

The benefit of mixing CH with CHX to improve the antibacterial property of CH as an intracanal medicament in elimination of *E. faecalis* remains a matter of controversy. The possible reasons for this controversy are the differences in the methods and materials used, including microbiological assessments (i.e. agar diffusion method, dentin block model etc.), concentrations and physical forms of CHX (i.e. gel, solution), time periods of experiments, strains and concentrations of *E. faecalis*, methods of bacterial inoculation, methods used for placing the medicaments, and depths of sampling.

Various methods have been used in order to define the antimicrobial effects of intracanal medicaments, such as dentin powder model, dentin block model, agar diffusion method, and broth dilution method. Agar diffusion is an *in vitro* model which has been the most commonly used technique[91]. However, it has some critical disadvantages, including "carry-over" effect, unknown reactions between agar plate ingredients and the antimicrobial agent, absence of a true correlation between the results of agar diffusion method and the *in vivo* environment, the buffering capacity of the agar plate compromising the capacity of antimicrobial agent, and absence of differentiation between bactericidal and bacteriostatic agents[33,91]. Therefore, agar diffusion method was considered as an exclusion criterion. Dentin powder is an *ex vivo* model that has also some disadvantages, including partial loss of microanatomical structure of the tooth and the difficulty to create microbial biofilm[33], therefore, it was also set as an exclusion criterion. Dentin block model, another *ex vivo* model, is the most standard method, and a statistical comparison is somehow feasible[34]. Penetration into dentinal tubules is the most important resistance mechanism of *E. faecalis* against antibacterial agents in endodontic

Studies	Exclusion criteria
Estrela, et al.[16] (2001)	2 & 6
Basrani, et al.[7] (2002)	5
Gomes, et al.[27] (2003)	3
Haenni, et al.[35] (2003)	2
Lin, et al.[52] (2003)	2
Zehnder, et al.[114] (2003)	6
Siren, et al.[95] (2004)	6
Zerella, et al.[115] (2005)	5
Onçag, et al.[68] (2006)	2
Oztan, et al.[71] (2006)	2 & 3 & 4
Gomes, et al.[28] (2006)	2
Ballal, et al.[6] (2007)	2
Wang Kou; Siguas Meneses[113] (2007)	4 & 5
Souza-Filho, et al.[97] (2008)	2
Vianna, et al.[109] (2008)	2 & 5
Gomes, et al.[26] (2009)	2
Ravishanker; Rao[82] (2009)	2
Aguiar[3] (2009)	5
Turk, et al.[102] (2009)	2
Valera, et al.[105] (2009)	5
Jhamb, et al.[40] (2010)	2
Mohammadi[62] (2010)	1
Gondim[29] (2010)	3 & 5
Oliveira, et al.[67] (2010)	2 & 3
Maekawa[56] (2010)	5
Valera, et al.[104] (2010)	5
Mohammadi; Dummer[63] (2011)	1
Silveira, et al.[89] (2011)	2
Gondim, et al.[30] (2012)	2 & 3
Lima, et al.[50] (2012)	3
Fedorowicz, et al.[22] (2012)	1
Maekawa, et al.[57] (2013)	5
Pacios, et al.[72] (2012)	2
Adl, et al.[2] (2012)	2
Mohammadi; Shalavi[64] (2012)	1
Atila-Pektaş, et al.[5] (2013)	4

1=Review article, 2=*In vitro* study, 3=Vehicles other than distilled water or saline for CH-alone group, 4=CH or CHX as medicament in other materials, 5=Any intervention except medicament dressing for bacterial elimination, 6=Qualitative results or invalid means and standard deviations (SD) reported

Figure 3- Excluded studies with reasons for exclusion

treatment[32,92]. This model provides reconstruction of the microanatomy of dentin, especially dentinal tubules. Dentin block model also simulates the chemical environment of the root canal and the ability of biofilm development[33], therefore, it was set as an inclusion criterion.

In general, three types of vehicles are used for preparing CH: aqueous, viscous, and oil[21]. The first group promotes a high degree of solubility when the paste remains in direct contact with tissues and tissue fluids[21]. The two other types result in the lower solubility and diffusion of the paste within the tissues[21]. In addition, some aqueous vehicles such as camphorated monochlorophenol have antibacterial effect on microorganisms, therefore, vehicles other than distilled water or saline solution for CH-alone group was set as exclusion criteria.

Since the evaluation of antibacterial effect of CH as an intracanal dressing was the aim of this meta-analysis, the use of antimicrobial irrigants in addition to the CH intracanal medication were considered confounding factors. Some relevant articles have presented this confounding factor as chemomechanical preparation of the canal before CH dressing and after microbial suspension inoculation into the canal[3,29,56,57,104,105,108,113,115]. In addition, one study has applied chemomechanical preparation after CH dressing[7], leading to the exclusion of these studies.

The time needed for CH to optimally disinfect the root canal system is still unknown and might be related to root canal exudate, the microorganism type, microorganism location in the root canal system, the smear layer, and the degree of susceptibility to the medication[28]. Although Shuping, et al.[88] (2000) reported that use of CH in the canals for 1 week resulted in a 92.5% reduction, evidence shows that CHX has antibacterial activity against *E. faecalis* after 1 day[27,34,45]. In addition, two studies showed that CH can be effective against enterococci after 24 hours[84,96]. Therefore, at least one day of dressing period was set as an inclusion criterion. Furthermore, the main dentinal structure of human and bovine teeth is not significantly different[32]. Therefore, the results of studies using both of them were used in the meta-analysis.

Evans, et al.[20] (2003) evaluated the antibacterial effect of CH/CHX mixture with two different depths of sampling, but they reported one mean and SD. Therefore, this study was considered as one comparison and was included in the meta-analysis. Another included study[4] was divided into nine comparisons according to different dressing periods and depths of sampling, but seven comparisons were excluded because of invalid means and standard deviations.

Qualitative data are not suitable for meta-analysis. Despite meeting all the inclusion criteria,

Table 1- Comparisons within 9 included studies

Reference	Sample size	Microbiologic unit	Depth (mm)	Dressing period (day)	Sig.	CHX type & concentration	Mean (SD)	
							CH	CH/CHX
Almyroudi, et al.[4] (2002)	16	CFU	0.1	14	0	1% Gel	2.70(2.90)	0.70(0.97)
Almyroudi, et al.[4] (2002)	16	CFU	0.35	14	0	1% Gel	1.57(1.85)	0.40(0.85)
Sukawat; Srisuwan[98] (2002)	12	OD	0.2	7	0	0.2% Sol	9.44(0.76*)	9.43(0.76*)
Sukawat; Srisuwan[98] (2002)	12	OD	0.35	7	0	0.2% Sol	9.70(3.80*)	9.75(3.80*)
Basrani, et al.[8] (2003)	30	OD	0.1	7	1	0.2% Gel	8.76(7.78*)	7.90(7.60*)
Basrani, et al.[8] (2003)	30	OD	0.2	7	1	0.2% Gel	8.83(7.60*)	7.95(7.48*)
Evans, et al.[20] (2003)	24	CFU	0.45	7	1	2% Sol	3.02(1.50)	1.36(1.61)
Lynne, et al.[55] (2003)	12	CFU	0.29	1	2	0.12% Sol	5.25(0.35*)	5.75(0.35*)
Lynne, et al.[55] (2003)	12	CFU	0.35	1	2	0.12% Sol	5.17(0.26*)	5.54(0.26*)
Lynne, et al.[55] (2003)	12	CFU	0.42	1	2	0.12% Sol	5.20(0.31*)	5.64(0.31*)
Schäfer; Bossmann[85] (2005)	10	CFU	0.05	3	0	2% Sol	2.14(0.26)	2.57(0.04)
Schäfer; Bossmann[85] (2005)	10	CFU	0.1	3	0	2% Sol	1.85(0.41)	1.93(0.16)
Schäfer; Bossmann[85] (2005)	10	CFU	0.15	3	0	2% Sol	1.43(0.18)	1.52(0.08)
Schäfer; Bossmann[85] (2005)	10	CFU	0.2	3	0	2% Sol	1.06(0.31)	1.36(0.14)
Ercan, et al.[15] (2006)	12	CFU	0.4	7	1	2% Sol	7.90(0.42*)	7.30(0.42*)
Ercan, et al.[15] (2006)	12	CFU	0.4	15	1	2% Sol	7.90(0.63*)	7.00(0.63*)
Ercan, et al.[15] (2006)	12	CFU	0.4	30	1	2% Sol	7.90(0.63*)	7.00(0.63*)
Delgado, et al.[14] (2010)	30	CFU	0.1	14	1	2% Gel	4.01(0.42)	0.50(0.35)
Delgado, et al.[14] (2010)	30	CFU	0.2	14	1	2% Gel	3.69(0.47)	0.77(0.44)
Perabhakal, et al.[80] (2012)	20	CFU	0.16	1	1	0.5% Sol	2.40(1.68)	2.05(1.35)
Perabhakal, et al.[80] (2012)	20	CFU	0.16	7	1	0.5% Sol	2.28(1.62)	1.87(1.16)

Sig.=Significance, CHX=Chlorhexidine, CH=Calcium Hydroxide, Sol=Solution, CFU=Colony Forming Unit, OD=Optical Density, *=estimated standard deviation, Sig. 0= no significant difference between CH and CH/CHX, Sig. 1= in favor of CH/CHX, Sig. 2= in favor of CH. SD= standard deviation.

two relevant studies[95,114] were excluded because they did not provide quantitative data. The results of four included studies[8,14,55,98] were illustrated in charts. Therefore, quantitative data were extracted from the illustrated charts using Adobe Photoshop software 5.0 (Adobe Systems, Mountain View, CA, USA) for Windows. To achieve more reliable data, a 300% zoom was used.

The greatest difference in the antibacterial activity of CH/CHX and CH groups has been reported in the study performed by Delgado, et al.[14] (2010). This might be due to longer dressing period, sample size, type and concentration of CHX compared with other studies.

In the present study, Cochran Q Test of Homogeneity showed that the 21 comparisons were

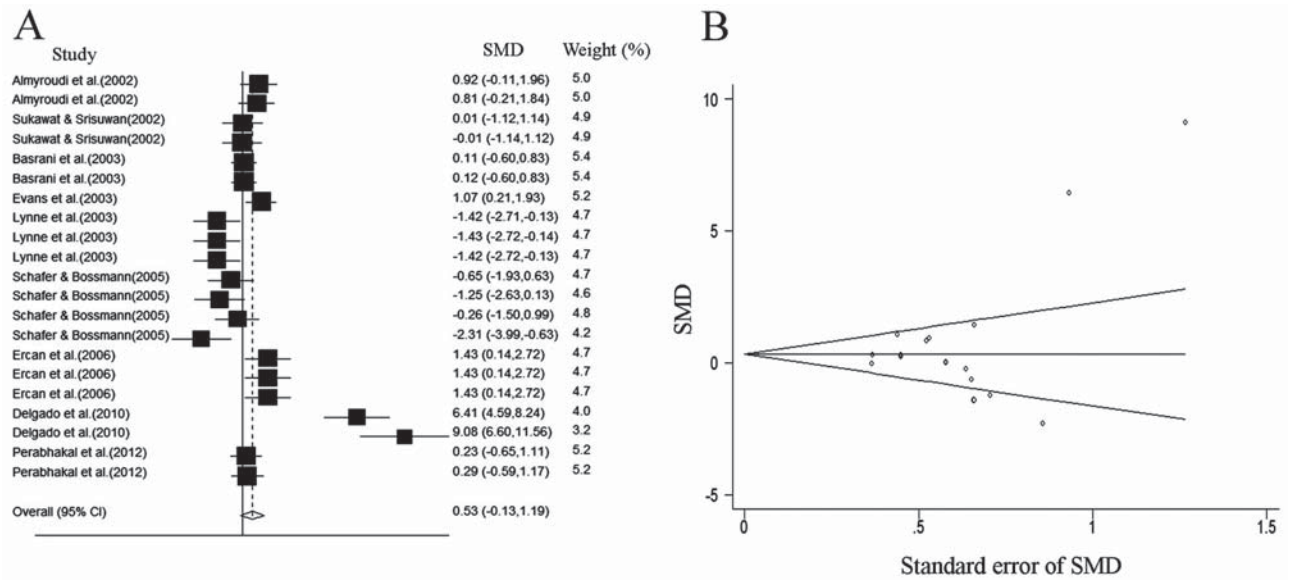

Figure 4- A: Forest plot for antibacterial effect of medicaments on *E. faecalis*. The box, its size and the horizontal line show the point of estimation, statistical weight and 95% confidence interval of each comparison, respectively. The diamond at the bottom of the figure illustrates the combined effect on the random effect model; B: Begg's Funnel Plot with 95% confidence limit for antibacterial effect of medicaments on *E. faecalis*. The plot shows a low risk of publication bias among the included articles. SMD: Standardized Mean Differentiation

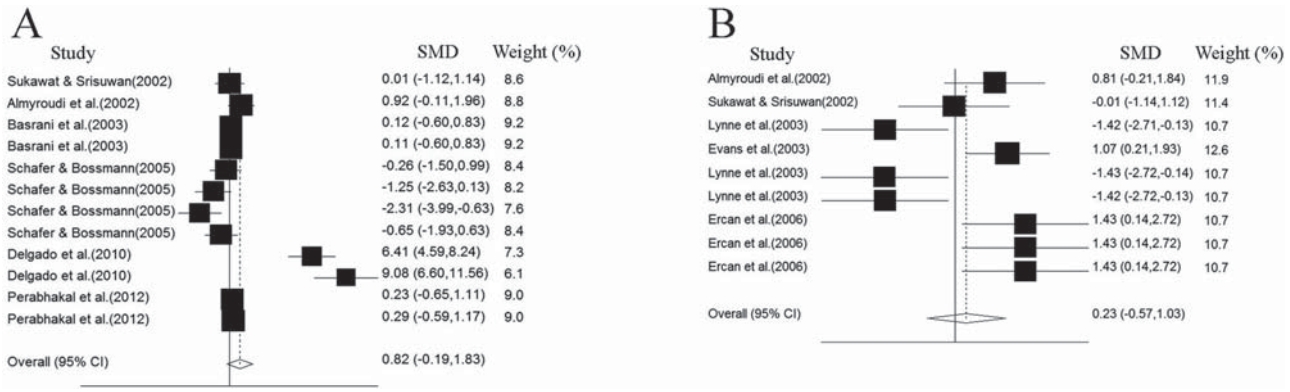

Figure 5- A and B: Forest plots for antibacterial effect of medicaments against *E. faecalis* in surface and deep dentin respectively. SMD: Standardized Mean Differentiation

heterogeneous. This might be due to differences in subjects (human or bovine dentin), method of medicament placement, dressing period, depth of sampling, and type and concentration of CHX.

Meta-analysis is a research tool designed to analyze and combine the inconsistent results of controversial subjects, particularly with those of randomized clinical trials. However, this method has been applied to *in vitro* studies[23,73,87,107]. Since there were no clinical trials on the subject of this systematic review, *in vitro* studies had to be selected. Therefore, only *ex vivo* dentin block model studies were selected, which have the greatest similarity to clinical conditions. This model, in comparison to other microbiological assessment models, is of high methodological quality and can simulate the clinical situation in the best way possible. On the other hand, the effectiveness of the medicament *in vivo* can be reduced by a variety of factors. These include problems in delivery, low overall volume, poor/incomplete penetration in the main root canal system, poor penetration into dentin, short contact time, or inactivation of the activity of the antibacterial agent by one or more of the chemical compounds present in the necrotic root canal.

The results of the present meta-analysis showed that CHX does not increase the antibacterial effect of CH. This may be due to deprotonation of CHX at high pH, which reduces its solubility and alters its interaction with bacterial surfaces as a result of the altered charge of the molecule[64].

In conclusion it appears that mixing CH with CHX does not improve its *ex vivo* antibacterial property as an intracanal medicament against *E. faecalis*. Further *in vivo* studies are necessary to confirm and correlate the findings of this study with the clinical outcomes.

ACKNOWLEDGMENTS

This study was supported by Isfahan University of Medical Sciences, Iran (grant no. 291230). The authors declare no conflict of interests.

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