Effects of Different Vehicles on The Antimicrobial Activity of Calcium Hydroxide

Farklı Taşıyıcıların Kalsiyum Hidroksitin Antimikrobiyal Aktivitesi Üzerine Etkisi

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Abstract

Objective: Calcium hydroxide (CH) has been widely used in endodontics as a root canal dressing. It has been reported that vehicles may have influence on the effectiveness of calcium hydroxide. The aim of this study was to evaluate the susceptibility of *Enterococcus faecalis* and *Candida albicans* to calcium hydroxide mixed with different types of vehicles.

Materials and Methods: Mixtures of glycerin or propylene glycol with deonized water at concentrations 20% and 100%, and mixtures of cetrimide with deonized water at 2%, anaesthetic solution and deionized water were used as vehicles. CH was added to the 5 different vehicles and saturated solutions were prepared. Antimicrobial effect of these samples was determined by the filter paper method.

Results: This study indicated that addition of Ca(OH)2 to the vehicles increased the antimicrobial effects. There were significant differences between 20% glycerin alone and the calcium hydroxide combination against to *E. faecalis* and 20% propylene glycol and deionized water alone and the calcium hydroxide combinations against to *C. albicans*.

Conclusion: The highest value of antimicrobial activity against to *E. faecalis* and *C. albicans* was obtained from 100% propylene glycol and 2% cetrimide alone.

Keywords: Calcium hydroxide, *Enterococcus faecalis*, *Candida albicans*.

Özet

Amaç: *Kalsiyum hidroksit (KH), endodontide kök kanal dezenfektanı olarak yaygın biçimde kullanılmaktadır. Kalsiyum hidroksitin kanal içi etkinliğinde kullanılan taşıyıcıların rolü bilinmektedir. Bu çalışmanın amacı; Enterococcus faecalis ve Candida albicans'ın farklı tipteki taşıyıcılar ile hazırlanmış kalsiyum hidroksit patlarına karşı hassasiyetlerini ortaya koymaktır.*

Gereç ve Yöntem: *Bu çalışmada gliserin ve propilen glikolün deiyonize su ile hazırlanan %20 ve %100'lük, setrimitin %2'lik konsantrasyonları ile anestezik solüsyon ve deiyonize su taşıyıcı olarak kullanıldı. Beş farklı taşıyıcıya KH ilave edilmiş ve doymuş solüsyonlar hazırlandı. Hazırlanan örneklerin antimikrobiyal etkileri filtre kağıt yöntemi kullanılarak saptandı.*

Bulgular: *Çalışmanın sonuçları göstermiştir ki; kullanılan taşıyıcıların tümüne kalsiyum hidroksitin ilave edilmesi, antimikrobiyal etkinliklerinde artışa yol açtı. Öte yandan, E. faecalis üzerine antimikrobiyal etki açısından sadece %20 gliserinin kalsiyum hidroksitle kombine kullanımı, tek başına kullanımına oranla anlamlı bir farklılık göstermiş, C. albicans için de, %20 propilen glikol ile, deiyonize suya kalsiyum hidroksitin ilavesi mevcut antimikrobiyal etkilerinde istatistiksel olarak da fark saptanmasına yol açmıştır.*

Sonuç: *Farklı taşıyıcılardan sadece ikisi; %100 propilenglikol ve %2 setrimit, tek başlarına kullanıldıklarında her iki mikroorganizma içinde en yüksek antimikrobiyal etkinliği göstermiştir.*

Anahtar Sözcükler: *Kalsiyum hidroksit, Enterococcus faecalis, Candida albicans.*

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Infection of the dental root canal system is considered as one of the major causes of apical periodontitis. Various studies have indicated that the outcome of endodontic treatment is often unsuccessful if bacteria are present in the root canal when it is obturated.[1,2] Therefore, one of the major goals of root canal treatment is the elimination of bacteria from the root canal system and the removal of the substrate on which they depend to prevent persistent root canal infection. This is traditionally achieved by mechanical instrumentation combined with various irrigating solutions, as well as antibacterial dressing of the canal between appointments.[3,4] For a longer period of time in the canal, intracanal dressing may penetrate into areas not reached by instruments and irrigants.[5] In addition, acting as a physical barrier, an intracanal dressing between appointments prevents both root canal re-infection and nutrient supply to remaining bacteria, thereby favoring periapical tissue repair.[5,6]

Nowadays, calcium hydroxide (CH) has been widely used in endodontics as an intracanal dressing based on its antibacterial, antiresorptive and tissue dissolving properties.[7,8] It has been shown that CH dressing in root canals can eliminate and prevent regrowth of bacteria.[9] Killing of bacteria by CH will depend on the ability to rapidly dissociate into hydroxyl (OH^-) and calcium ions (Ca^{++}), which then diffuse into the surrounding tissues.[10] The vehicle plays a most important role in the overall process, because it determines the velocity of ionic dissociation causing the paste to be solubilized and resorbed at various rates by the periapical tissues and from within the root canal.[8] Some *in vitro* studies have shown that the type of vehicle has a direct relationship with the antibacterial action.[8] Surface active agents reduce the surface tension of the substances which they are mixed with, and also increase the microbial effects of most of the antiseptic agents. A quarternery ammonium compound, a cationic detergent and a surface active agent cetrimide (cetiltrimethylammonium bromide) is effective against most of the Gram (+) and Gram (-) bacteria.[11] In a previous study, Pehlivan *et al.*[12] showed that, the lowest surface tension values were observed in both CH+100% propylene glycol and CH+ 2% cetrimide mixtures.

The bacterial flora in root canal infections is polymicrobial with a strong dominance of anaerobic bacteria.[2,13] Persistent endodontic infections are often caused by *Enterococcus faecalis*. This bacterium has been reported to survive long periods without nutrients and the most resistant to CH of the species tested.[13] Microbiological investigations have also shown that yeast can be isolated from therapy-resistant cases of apical periodontitis.[14] The incidence of yeasts in infected root canals is reported to vary between 7% and 55%.[15] It has been concluded that presence of yeasts, particularly in root canals, may represent a reservoir for dissemination of the disease to the periphery by means of the bloodstream.[15]

Therefore, the aim of this study was to evaluate the susceptibility of *Enterococcus faecalis* and *Candida albicans* to calcium hydroxide mixed with different types of vehicles including cetrimide.

Materials and Methods

Samples were prepared by mixing CH powder (Merck; Merck, Germany) with 5 different vehicles. These vehicles were glycerin (Sigma, St Louis, MO), dental anaesthetic solution (Citanest-Octapressin 3% Astro, Sweden), 1,2 propylene glycol (Merck), cetrimide (Drogsan, Ankara, Turkey), and deionized water.

Preparation of the samples

Mixtures (25 ml) of glycerin or propylene glycol with deionized water at concentrations 20% and 100%, and 2% concentration of cetrimide were prepared in 50 ml conical centrifuge tubes. CH was added to the solutions (0.3 g) and saturated solutions were prepared by shaking tubes (Veb Electroindustrie Postdam, Germany, 200 rpm; 90 min). The undissolved CH was then separated by centrifugation (Denley BS400, England, 3000 rpm; 10 min). The solutions which were tested are shown in Table 1.

Table 1. The test solutions

20% Propylene glycol (Merck,Germany)
20% Propylene glycol + CH
100% Propylene glycol (Merck,Germany)
100% Propylene glycol + CH
2% Cetrimide (Drogsan,Ankara)
2% Cetrimide + CH
20% Glycerin (Sigma, St Louis)
20% Glycerin + CH
100% Glycerin (Sigma, St Louis)
100% Glycerin + CH
Anesthetic solution (Citanest-Octapressin 3%Astro, Sweden)
Anesthetic solution + CH
Deionized water
Deionized water + CH

Determination of antibacterial activity

A loopful of each microorganism stock culture was incubated in 5 mL Tryptone Soya Broth (TSB, Oxoid, Hampshire, England) at 37°C overnight. Aliquots of 0.5 mL of each suspension were then added to 100 mL of TSB broth and further incubated at 37°C for 12h. The suspension was spectrophotometrically standardized to a final cell density of approximately 1×10^{8} cfu/mL[-1]. Sterilized filter paper discs (Oxoid, antimicrobial susceptibility test discs: 4 mm diameter) were immersed in the microorganism suspension at room temperature for 10 min. The filter paper discs were removed from the suspension and drained of excess liquid. The discs were placed into test tubes containing 2 mL TSB with 3 mm diameters 10 glass beads and 1 mL test solution. Test solutions were incubated at 37°C for 1h, after discs were placed into 2 mL TSB with 3 mm diameters 10 glass beads which were vigorously shaken on a Vortex mixer for 30 s. The TSB and test solutions with re-suspended microorganisms were serially diluted 10-fold. Droplets of 25 µL from each dilution were inoculated on TSB agar plates and incubated in air at 37°C for 24 h. The number of cfu was then calculated from the counts at the appropriate dilutions. The test was repeated triplicate. Statistical evaluation of the results was performed with one-way analysis of variance (ANOVA) followed by the Dunnett C Multiple comparison post-hoc test ($p = 0.05$).

Results

The results of the filter paper test are listed in Table 2. There were significant differences between the vehicles. The microbial activity of vehicles alone showed that deionized water had the lowest value, whereas the highest value was obtained from 100% propylene glycol and 2% cetrimide. Compared with *E. faecalis*, *C. albicans* showed higher resistance to 20% propylene glycol, 20% glycerin, 100% glycerin, anaesthetic solution and deionized water. Although effectiveness of vehicles increased with decrease of water rate, there was no statistically significant difference ($p>0.05$). The addition of CH to the vehicles was resulted in an increase in the microbial activity. But this increase was found statistically significant only in 20% propylene glycol and deionized water and 20% glycerin. The increase of propylene glycol and glycerin concentrations from 20% to 100% did not have any effect on microbial activity and were not found statistically significant ($p>0.05$).

Table 2. Survival of *C. albicans* ATCC 10239 and *E. faecalis* after incubation for 1 h in vehicles alone and vehicle - CH solutions as measured by filter paper test.

	C. albicans ATCC 10239	*E. faecalis* ECOC 80
20% Propylene glycol	1.73×10^{6} (31.46%)	0
20% Propylene glycol - CH	0	0
100% Propylene glycol	0	0
100% Propylene glycol - CH	0	0
2% Cetrimide	0	3×10^{2} (0.01%)
2% Cetrimide - CH	0	0
20% Glycerin	1.23×10^{6} (22.37%)	9.3×10^{5} (14.31%)
20% Glycerin - CH	8.3×10^{4} (1.51%)	5.7×10^{2} (0.01%)
100% Glycerin	5×10^{3} (0.1%)	1.3×10^{5} (2.31%)
100% Glycerin - CH	2×10^{4} (0.37%)	2×10^{2} (0.01%)
Anesthetic solution	3×10^{5} (5.56%)	3.6×10^{3} (0.06%)
Anesthetic solution - CH	0	0
Deionized water	4.6×10^{6} (85.64%)	2×10^{6} (36.36%)
Deionized water - CH	2.1×10^{4} (0.39%)	1.5×10^{2} (0.01%)

Discussion

It has been asserted that all biological actions of CH will be progressed by the ionic dissociation in Ca^{2+} and OH^{-} ions.[8] CH kills bacteria because of the effects of hydroxyl ions.[15] These ions are extremely reactive, combining rapidly with lipids, proteins, and nucleic acids. They cause lipid peroxidation, increasing the bacterial membrane permeability; protein denaturation, inactivating enzymes; and DNA damage. These procedures can cause bacterial death.[10] Nevertheless; killing of bacteria by calcium hydroxide will depend on the availability of hydroxyl ions in the paste. Some *in vitro* studies have shown that the type of vehicle has a direct relationship with the antimicrobial action of CH based paste.[8] For CH to act effectively as an intracanal dressing to perform its bactericidal function, besides the dissociation of hydroxyl ions into the environment, these hydroxyl ions must be able to diffuse through the dentin and the uninstrumented areas of the root canal system. Spreading of the disinfectant into the dentinal tubules depends on its surface tension.[12] Esberard *et al.*[17] reported that the surface tension of the liquid vehicle may have an influence on diffusion of the hydroxyl ions. Our previous study showed that the lowest surface tension values were observed in both CH+100% propylene glycol and CH+2% cetrimide mixtures.[12] According to the results of current study, 100% propylene glycol and 2% cetrimide alone and CH+100% propylene glycol and CH+2% cetrimide mixtures also showed the highest antibacterial activity against to *E. faecalis* and *C. albicans*.

E. faecalis was selected as a test microorganism in the present study because it is one of the most resistant microorganisms in root canal flora. This strain, a facultative anaerobe, can grow in aerobic conditions and can be handled and cultured easily.[18] CH solutions used in this study were effective in the elimination of *E. faecalis*. This result was in agreement with the observations of Han *et al.*[18] But it was not in agreement with the observations of Heling *et al.*[9] who reported less bacterial activity of CH against *E. faecalis* in the dentinal tubules of bovine teeth. The difference between our study and the previous studies may result from the test material used.

The literature concerning the susceptibility of yeasts to CH is very limited. Waltimo *et al.*[19] showed that *C. albicans* was the most common species isolated from root canals.[14] *C. albicans* is often present also in the oral microflora of healthy individuals.[19] All *Candida* species are either similarly or more resistant to CH than *E. faecalis*, which has been reported to be the most resistant microorganism among bacteria tested *in vitro*.[14] In the present study; whereas *C. albicans* was more resistant to the vehicles alone than *E. faecalis*, addition of CH to the vehicles affected two microorganisms similarly. Barbosa *et al.*[20] studied the effect of aqueous CH solution and combinations with detergents on microorganisms. The *C. albicans* strain was susceptible to CH and was killed after incubation for less than 5 min. Differences in results might have been due to differences in the experimental methods employed.

In the present study, antimicrobial effect of CH was determined by the filter paper method. This method is suitable for testing susceptibility of yeasts to disinfectants.[14] Immersion of the filter paper disc in a standardized yeast suspension gave a well-standardized inoculum formed by a homogenous layer of yeast cells adsorbed on the surface of the filter paper disc.[14] According to our data; *C. albicans* was more resistant than *E. faecalis* to the test solutions. When the filter paper discs were immersed in deionized water; *E. feacalis* transported away from disc to deionized water more easily because of its smaller size than *C. albicans*. Therefore we could observe 16% decrease in *C. albicans* and 63% in *E. faecalis* in the deionized water alone; in spite of the fact that deionized water has not antibacterial activity. In the glycerin groups; antimicrobial activity was found higher in the 100% glycerin than 20%. According to our knowledge, glycerin does not have antimicrobial activity. Therefore, decreasing cell number might depend on only the hypertonic structure of glycerin. Intracellular fluid leaks out of the cell wall, because of the hypertonic structure of glycerin and this may leak to the cell death.

As we know, oral *Candida* strains were resistant to a commonly used local root canal medicament, CH, *in vitro*.[16] It is common that oral *Candida* isolates are more prone to primary and secondary defense mechanisms.[15] In addition to the host defenses, environmental changes such as pH, temperature, and nutritional source also challenge the *Candida* genus.[15] Under this conditions, micromorphologic and physiologic properties of *C. albicans* are rapidly modified, and thus, phenotypic switching occurs in a particular ecological niche.[15] Phenotypic switching and resistance mechanisms may explain the resistance to disinfecting solutions.[15] The correlation between *in vitro* and *in vivo* susceptibility of yeasts to CH is not known. Nevertheless, the *in vitro* resistance may partly explain the isolation of yeasts from persisted apical periodontitis. And it is clear that there is a need for effective medication for yeasts. According to results of this study, the highest value of antimicrobial activity was obtained from 100% propylene glycol and 2% cetrimide alone. Although the addition of CH to the all of the vehicles resulted in an increase in the antimicrobial activity against to *E. faecalis* and *C. albicans*, according to our previous study, because of 100% propylene glycol and 2% cetrimide's lowest surface tension values and 2% cetrimide's highest conductivity value, 2% cetrimide seems to be the best choice as a vehicle for CH.

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