Antimicrobial Substantivity of Cavity Disinfectants

Kavite Dezenfektanlarının Antimikrobiyal Etkinlik Sürekliliği

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Abstract

Objective: The aim of this study was to examine the antimicrobial substantivity of different disinfectants comparatively.

Methods: In this study, 2% chlorhexidine gluconate, 2% benzalkonium chloride, Tubulicid Red, Consepsis and 5.25% sodium hypochlorite (NaOCl) were tested as cavity disinfectants. One hundred ant thirty five enamel-dentin discs were used with agar diffusion method to test antimicrobial substantivity of disinfectants against; *Streptococcus mutans, Lactobacillus acidophilus* and *Candida albicans*. Statistical analysis was carried out using 4x5 factorial ANOVA and an analysis of variance with Duncan's test of multiple comparisons.

Results: Two percent of chlorhexidine gluconate was effective against all bacteria for 96 hours. Consepsis was inhibitory against *S. mutans* at the 96-hour time period, whereas its activity on the other bacteria continued for 72 hours. Antimicrobial activity of 2% benzalkonium chloride continued against all bacteria for 72 hours. At the same period, Tubulicid Red showed activity against only *S. mutans*. NaOCl was effective against all bacteria only for 24 hours.

Conclusion: Chlorhexidine-based solutions showed the most effective and longer lasting antimicrobial activity in this study.

Keywords: Chlorhexidine gluconate, benzalkonium chloride, cavity disinfectant

Özet

Amaç: *Bu çalışmanın amacı, farklı kavite dezenfektanlarının antimikrobiyal etkinliklerinin sürekliliğini karşılaştırmalı olarak incelemektir.*

Yöntem: *Bu çalışmada kavite dezenfektanı olarak, klorheksidin glukonat ve benzalkonyum klorürün %2'lik çözeltileri, Consepsis, Tubulicid Red ve % 5.25'lik sodyum hipoklorit (NaOCl) test edildi. Yüz otuz beş mine-dentin diski agar difüzyon yöntemi ile birlikte, dezenfektanların Streptococcus mutans, Lactobacillus acidophilis ve Candida albicans'a karşı antibakteriyel etkinlik sürelerinin test edilmesi için kullanıldı. Verilerin istatistiksel analizi ANOVA, 4x5 faktöryel ANOVA ve Duncan testleri kullanılarak yapıldı.*

Bulgular: *Yüzde ikilik klorheksidin glukonat solüsyonu 96 saat süresince tüm bakterilere karşı etkiliydi. Consepsis, 96 saat zaman periyodunda sadece S. mutans'a karşı etkinlik gösterirken, diğer bakteriler üzerindeki etkisi 72 saat boyunca devam etti. Yüzde ikilik benzalkonyum klorürürün antibakteriyel aktivitesi tüm bakterilere karşı 72 saat devam etti. Aynı zaman diliminde, Tubilicid Red sadece S. mutans'a karşı etki gösterdi. NaOCl ise tüm bakterilere karşı sadece 24 saat etkili olabildi.*

Sonuç: *Bu çalışmada, klorheksidin içerikli solüsyonlar en etkin ve uzun süreli antibakteriyel aktivite gösterdiler.*

Anahtar sözcükler: *Klorheksidin glukonat, benzalkonyum klorür, kavite dezenfektanı*

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Introduction

Dental caries is a pathological process of localized destruction of tooth tissues by the activity of microorganisms on fermentable carbohydrates in dental plaque. Restorative procedures such as cavity preparation are used to remove the infected dentin and to make space for restorative materials. Success criteria of these procedures are mainly due to the effective removal of infected dentin prior to placing a restorative material. It is known that pulp infection that occurs after the placement of restoration depends on the bacterial activity in the prepared cavity rather than toxic effects of restorative materials. Thus, elimination of bacteria in a prepared cavity is one of the most important procedures during restorative treatments. However, the procedures used in the treatment of caries do not always eliminate all of the microorganisms in residual tissues.[1] A number of studies have demonstrated that the bacteria left in the dentin of the cavity could maintain their activity for a long time.[2] Bacteria invading the tooth/restoration interface are known to be the main cause of pulpal inflammation, pulp sensitivity and secondary caries.[3] The persisting bacterial presence and leakage along the interface between the filling and cavity walls may be involved in development of recurrent caries and subsequent pulpal inflammation. Therefore, usage of an antibacterial solution after cavity preparation has been recommended to disinfect dentin.[4]

Previous studies have shown that a number of solutions can be used as cavity disinfectants.[3-6] The current generation of disinfectants contains 2% chlorhexidine gluconate as the primary active ingredient in addition to benzalkonium chloride.[7] While the majority of the investigations have focused on the immediate antimicrobial action of the disinfectants,[6] antimicrobial substantivity is also one of the desired properties of a cavity disinfectant.[5,8,9] The ability of drugs to adsorb onto and bind to soft and hard tissues is known as substantivity.[10] The slow release of the drug from the tissues provides a prolonged antimicrobial effect.[11] If the disinfectant maintains its activity for a long time period, it may provide more effective antimicrobial protection. Furthermore, prolonged antimicrobial activity is particularly important when the bacterial leakage occurs from the tooth surface via marginal gap formation between a tooth and restorative material.

The purpose of this study was to examine the antimicrobial substantivity of different disinfectant solutions comparatively.

Materials and Methods

Disinfectant solutions tested in this study and their active ingredients are shown in Table 1. Antimicrobial activity of each disinfectant was evaluated against the following bacteria provided from the stocks of the Department of Basic and Industrial Microbiology, School of Science, Ege University: *Streptococcus mutans* CCUG 6519, *Lactobacillus acidophilus* LA-CH-5 DVS, *Candida albicans* ATCC 10239.

All bacteria were cultured overnight at 37°C in yeast-glucose broth (concentration of glucose extract 2%; yeast extract 2%) (Difco, Detroit, MI, USA). Then, the broth culture was diluted ten fold, and grown as above to a density of 10^6 colony forming units (cfu)/ml which was confirmed by viable cell count and by optical density using a 550 nm spectrophotometer (Cary 50-Bio UV-visible spectrophotometer, Mulgrave, Australia). Brain-Heart Infusion Agar (Difco) was performed into agar plates. Five wells (2 mm in depth and 4 mm in diameter) were punched equidistantly in each agar plate using a sterile steel punch. The bacteria were swabbed over the surfaces of the agar plates in two directions by the cotton-tipped applicator.

Table 1. Disinfectant solutions used in this study.

Product	Active ingredients	Manufacturer
Laboratory made	2% Benzalkonium chloride	Gem San, Istanbul
Laboratory made	2% Chlorhexidine gluconate	Drogsan, Ankara
Consepsis	2% Chlorhexidine gluconate	Ultradent, Utah, USA
Domestos	5.25% Sodium hypochlorite	Lever, Istanbul
Tubulicid Red	0.1% Benzalkonium chloride 0.2% EDTA, 1% Sodium fluoride	Global Dental, Malmö, Sweden

Antimicrobial effects of cavity disinfectants were tested using enamel-dentin discs. One hundred and thirty five enamel-dentin discs obtained from human maxillary and mandibular premolar teeth were used. The enamel dentin discs were cut perpendicular to long axis of the teeth above the pulp chamber after the occlusal surfaces were removed, so as to expose the dentin using a slow-speed diamond saw (Isomet, Buehler Ltd, Evanston, IL, USA) under copious water spray. The discs were 2±0.5 mm in height and 4±0.5 mm in diameter. The outer surface of the enamel, where the diameters of the discs exceed 4±0.5 mm, was abraded with a No. 956UF.314.010 diamond-finishing bur (Komet, Lemgo, Germany) in a high-speed handpiece with water coolant until the proper dimensions was obtained. In order to remove smear layer, all surfaces of the enamel-dentin discs were etched with 35% phosphoric acid (3M Dental Products, St. Paul, MN, USA) for 15 seconds, rinsed with water for 15 seconds and dried with compressed air for 10 seconds. The prepared enamel-dentin discs were autoclave sterilized (at 121°C for 15 min) before application of the disinfectant.

The disinfectants were applied for 60 seconds with a sterile brush applicator thoroughly in order to treat all surfaces of the enamel-dentin discs. The discs were then air dried and placed on a sterile blotter paper. Immediately after the inoculation of the plates, the treated enamel-dentin discs were placed in the prepared wells. All procedures were carried out under aseptic conditions in a laminar airflow cabinet. All plates were incubated aerobically at 37°C for 24 hours. After incubation, the diameter of the inhibition zone was measured in millimeters in two locations with a dial caliper, and the average calculated after the substraction of disc diameters (4 mm) from inhibition zone diameters.

In order to determine the residual antimicrobial activity of the disinfectants, the treated enamel-dentin discs were removed from the agar plates and transferred to fresh inoculated plates. This process was repeated four times at 24 hour-intervals until disinfectant solutions lost their antimicrobial activities. Statistical analysis was carried out using 4x5 factorial ANOVA (Completely Randomized Design) and an analysis of variance with Duncan's test of multiple comparisons. The level of significance was set as $p<0.05$.

Results

While disinfectants used in this study demonstrated antimicrobial activity against all three bacteria, there were statistically significant differences among them ($p<0.05$). The diameters of the inhibition zones produced by each disinfectant at different time periods are shown in Table 2.

When the antimicrobial activity of the cavity disinfectants against *Streptococcus mutans* was evaluated throughout all time periods; 2% benzalkonium chloride, Consepsis and 2% chlorhexidine gluconate showed the most effective inhibitory activity, followed by Tubulicid Red. NaOCl had less inhibitory effect on *Streptococcus mutans* (Graph 1).

In all time periods, 2% chlorhexidine gluconate had the highest inhibitory effect on both *Lactobacillus acidophilus* and *Candida albicans* ($p<0.05$). Antimicrobial activities of 2% benzalkonium chloride and Consepsis were less effective than that of 2% chlorhexidine gluconate, but more effective than those of Tubulicid Red and NaOCl (Graphs 2 and 3).

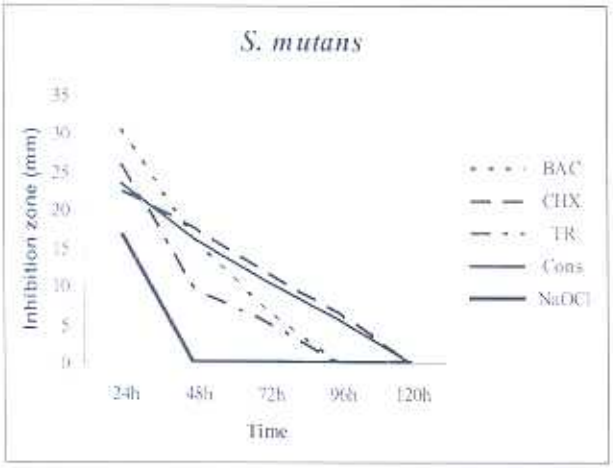
Inhibitory activity of 2% chlorhexidine gluconate was effective against all bacteria for 96 hours. Consepsis showed effective inhibitory activity only against Streptococcus mutans at the 96-hour time period, whereas its activity on *Lactobacillus acidophilus* and *Candida albicans* continued for 72 hours.

Two percent of benzalkonium chloride was effective against all three bacteria for 72 hours. At the 72-hour time period, Tubulicid Red showed activity only against *Streptococcus mutans* and its activity against other bacteria continued for only 48 hours. NaOCl showed antimicrobial activity against all three bacteria only for 24 hours. None of the disinfectants showed antibacterial activity against all three bacteria used in this study at 120-hour time period.

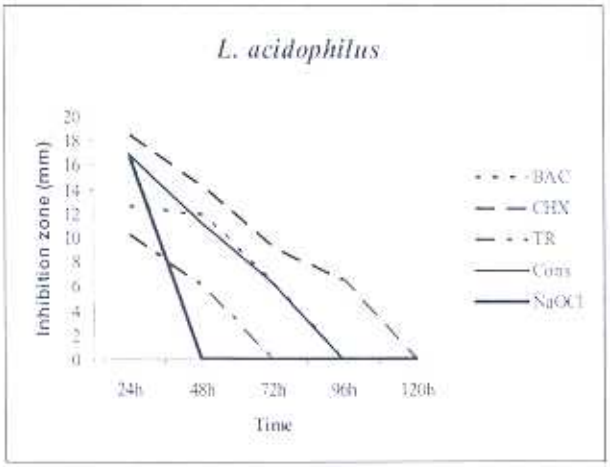
Table 2. Diameter of inhibition zones in millimeters produced by each disinfectant against all three bacteria (Mean±SD) (n=9).

TIME	DISINFECTANT	*S. mutans*	*L. acidophilus*	*C. albicans*
24- Hour	2% Benzalkonium chloride	30.40±0.70[a*]	12.61±0.50[d]	16.36±0.17[a]
	2% Chlorhexidine gluconate	22.56±0.52[b]	18.55±0.36[b]	15.60±0.25[b]
	Consepsis	23.64±0.50[b]	16.96±0.44[c]	15.99±0.44[b]
	5.25% NaOCl	16.36±0.41[d]	16.40±0.33[d]	13.80±0.55[c]
	Tubulicid Red	25.91±0.67[c]	10.26±0.37[e]	10.73±0.67[d]
48- Hour	2% Benzalkonium chloride	15.91±0.58[a]	11.95±0.12[a]	13.32±0.41[a]
	2% Chlorhexidine gluconate	17.70±0.27[b]	14.38±0.23[b]	14.25±0.29[b]
	Consepsis	16.10±0.50[a]	11.24±0.89[a]	11.51±1.17[c]
	5.25% NaOCl	-	-	-
	Tubulicid Red	9.68±0.64[c]	6.04±1.25[c]	7.08±0.50[d]
72- Hour	2% Benzalkonium chloride	6.85±0.22[a]	6.34±0.36[a]	7.77±0.67[a]
	2% Chlorhexidine gluconate	11.61±0.38[b]	9.20±0.64[b]	10.58±0.33[b]
	Consepsis	10.63±0.29[c]	6.35±0.98[a]	6.52±0.45[c]
	5.25% NaOCl	-	-	-
	Tubulicid Red	5.39±0.81[d]	-	-
96- Hour	2% Benzalkonium chloride	-	-	-
	2% Chlorhexidine gluconate	6.58±0.26[a]	6.54±0.48[a]	7.25±0.55[a]
	Consepsis	5.67±0.81[b]	-	-
	5.25% NaOCl	-	-	-
	Tubulicid Red	-	-	-

*Different letters in the same column for each time period indicate statistically significant differences (p<0.05).

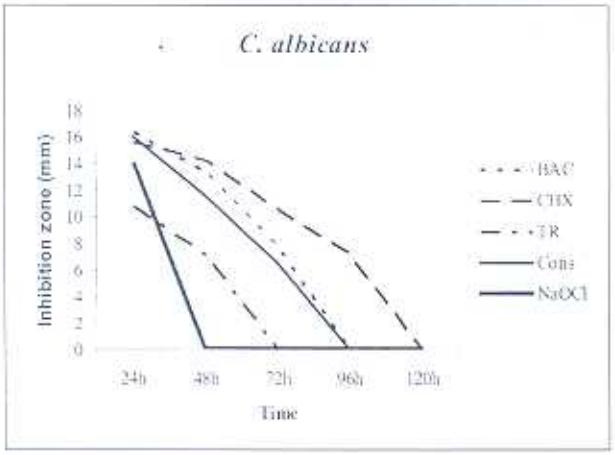


Graph 1. Antibacterial activity of disinfectants against *Streptococcus mutans*.



Graph 2. Antibacterial activity of disinfectants against *Lactobacillus acidophilus*.

*BAC (Benzalkonium chloride), CHX (Chlorhexidine gluconate), TR (Tubulicid Red), Cons. (Consepsis), NaOCl (Sodium hypochlorite).



Graph 3. Antibacterial activity of disinfectants against *Candida albicans*.

Discussion

The agar diffusion test is generally an accepted method to test antimicrobial activity of dental materials. Placement of the material to be tested in well that was punched in the agar plate is a commonly used technique.[6,12] For this method, the zones of growth inhibition provided by the materials depend on the toxicity of the material against the bacteria tested. Although this method is simple, low-priced and can be re-applied easily, substrate's pH, incubation period, and the capacity of diffusion of disinfectant into the culture medium may affect antimicrobial activities of tested materials that have placed in agar plates.[13] Gultz et al.[6] have previously described the using of treated enamel-dentin discs with disinfectant solution in agar diffusion test to determine the antibacterial activity of disinfectant. In the present study, this method was used to investigate the antimicrobial substantivity of the cavity disinfectants. The enamel-dentin discs were placed in new agar plates at 24-hour intervals until they lost their antimicrobial activity. In a previous study, Ribeiro and Ericson[12] have used the similar method to assess the antimicrobial substantivity of chlorhexidine gluconate-containing glass ionomer cements.

In this study, the residual antimicrobial activities of disinfectans were examined against *Streptococcus mutans*, *Lactobacillus acidophilus* and *Candida albicans*. The first two microorganisms are thought to be involved with recurrent human caries.[14] Candida albicans is found in 35-40 % of adult oral cavities and was selected to evaluate the effects of the antimicrobial agents on eukaryotic cells.[15]

Chlorhexidine gluconate, particularly due to its substantive antimicrobial properties, has been recognized as an effective oral antimicrobial agent for use in periodontal therapy and caries prevention and as a therapeutic agent for oral infections in general.[11] Chlorhexidine gluconate is a broad-spectrum antibacterial agent and its use has been generalized over the past two decade for the chemical control of bacterial plaque and prevention of the dental caries. Positively charged molecules of chlorhexidine gluconate can adsorb onto the dentin[16] and prevent microbial colonization on the dentin surface for some time.[17] Although its initial activity was decreased in time, 2% chlorhexidine gluconate solution used in this study showed effective inhibitory activity against all three bacteria for 96 hours. White et al.[8] have reported that residual antimicrobial activity remained throughout the 72-hour testing period when root canals were irrigated with 2% chlorhexidine gluconate during the mechanical preparation. However, residual activity continued only for 6 to 24 hours after irrigation with 0.12% chlorhexidine gluconate. Komorowski et al.[9] used bovine dentin root model for testing the antimicrobial substantivity and demonstrated that treatment of dentin with 0.2% chlorhexidine gluconate for 7 days did induce antimicrobial substantivity over a period of 21 days, whereas 5-min treatment failed. The results of these two studies have indicated that substantivity is influenced by the concentration of the disinfectant and the contact period of the disinfectant with dental structures. In our study, the contact period of the cavity disinfectants with enamel-dentin discs was 60 seconds. If the treatment time was longer than 60 seconds, antimicrobial substantivity would have probably been longer.

Consepsis demonstrated less effective and shorter lasting antimicrobial activity (except against *Streptococcus mutans*) than 2% chlorhexidine gluconate solution. The reason for the difference between the results of two disinfectants may

depend on its higher viscosity, because viscous solutions can not diffuse into dentin disc easily. Sixty-second application-time might not be enough for the complete absorption of Consepsis by dentin discs. Substantive antimicrobial activity imparted by chlorhexidine gluconate is known to depend on the amount of chlorhexidine gluconate molecules available to interact with dentin.[18]

When Brännström and Nyborg[4] had first proposed the concept of disinfecting teeth, the recommended agent was a benzalkonium chloride-based disinfectant, Tubulicid. Benzalkonium chloride is a quaternary ammonium compound and bactericidal against gram-positive and some gram-negative bacteria, with little or no effect against tubercle bacilli and spore-forming microbes.[19] Brännström and Nyborg[20] had reported that no bacteria were detected on the cavity walls and no pulpal reactions were observed when the cavities had been treated with Tubulicid before placement of a restoration. In a recent study, Gultz et al.[6] demonstrated that chlorhexidine gluconate-based cavity disinfectant had the most effective antibacterial activity and was followed by benzalkonium chloride-based disinfectant, Tubulicid Red. Chan and Lo[5] have suggested that benzalkonium chloride also has the residual antimicrobial activity as chlorhexidine gluconate. In the present study, benzalkonium chloride-containing disinfectants had long lasting antimicrobial activities. Antimicrobial activity of 2% benzalkonium chloride was effective for 72 hours against all three microorganisms, while Tubulicid Red's activity continued for 72 hours against *Streptococcus mutans* and 48 hours against *Lactobacillus acidophilus* and *Candida albicans*. Tubulicid Red contains 0.2% ethylenediamine-tetraacetic acid for the removal of the smear layer, and a high concentration (1%) of sodium fluoride in addition to 0.1% benzalkonium chloride.[21] Lower benzalkonium chloride content of Tubulicid Red may be responsible for its lower and shorter lasting antimicrobial activity in comparison to 2% benzalkonium chloride.

The antibacterial effectiveness of NaOCl is well documented.[22] The germicidal ability of NaOCl stems from the formation of hypochlorous acid when in contact with organic debris. Hypochlorous acid exerts its effect by the oxidation of sulfhydryl groups of bacterial enzyme systems thereby disrupting the metabolism of the microorganism.[23] The use of NaOCl as a cavity disinfectant has been controversial because it removes the collagen layer and prevents hybridization.[24] Since its antimicrobial effect has been proven, 5.25% NaOCl has been adopted as a positive control for studies on bacterial growth or antibacterial activity.[25] Previous studies have shown that NaOCl may be effective on initial exposure, but it is not a substantive antimicrobial agent.[8] Also in our study, 5.25% NaOCl did not show substantive antimicrobial activity in the gel form. If the solution form of sodium hypochlorite had been used, its antimicrobial substantivity could have been longer.

Conclusion

According to the findings and within the limitations of this study, it can be concluded that among the tested disinfectants, chlorhexidine-based solutions appeared to have the most effective and longer lasting antimicrobial activity. Benzalkonium chloride-based solutions also showed substantive antimicrobial activity, but their antimicrobial activities were more limited than chlorhexidine gluconate based disinfectants. It should be noted that this study was performed under *in vitro* conditions and direct extrapolation of a clinical situation must be done with caution.

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