

Antimicrobial and physical characteristics of orthodontic primers containing antimicrobial agents

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ABSTRACT

Objective: To compare the antimicrobial and physical properties of experimental primers containing chlorhexidine (CHX) or ursolic acid (UA) with a commercial primer.

Materials and Methods: Two antibacterial agents, 3 mg each of CHX and UA were incorporated respectively into 1 ml of Transbond XT primer (TX) to form antibacterial primers, TX-CHX and TX-UA. The antimicrobial activity of the three primers (TX, TX-CHX, and TX-UA) against *Streptococcus mutans* in both planktonic and biofilm phases was analyzed by determining minimum inhibitory and bactericidal concentrations and by performing growth and biofilm assays. Growth and biofilm assays were performed in both the absence and presence of thermocycling in a water tank to analyze the effects of water aging on the antimicrobial activities of primers. After bonding brackets onto bovine incisors using the primers, shear bond strength and mode of fracture were analyzed to compare physical properties.

Results: TX-CHX had stronger antimicrobial activity against *S. mutans* in the planktonic and biofilm phases than did TX or TX-UA. When applied, TX-CHX completely inhibited the growth and biofilm formation of *S. mutans*. In addition, the antimicrobial activity of TX-CHX was maintained after thermocycling. However, TX-UA did not show significant antimicrobial activity compared with TX. There was no significant difference in either shear bond strength or bond failure interface among the primers.

Conclusion: Incorporation of CHX into an orthodontic primer may help prevent enamel demineralization around surfaces without compromising its physical properties. (*Angle Orthod.* 2017;87:307–312)

KEY WORDS: Antimicrobial; Orthodontic primer; Bonding; Bond strength

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INTRODUCTION

During orthodontic treatment with fixed appliances, enamel demineralization commonly occurs at the interface between the bracket and the tooth surface. This is primarily caused by adhesion of cariogenic bacteria, such as *Streptococcus mutans*.¹ Many efforts have been made to develop an antibacterial orthodontic adhesive system to prevent enamel demineralization at the interface. One approach is to incorporate various antimicrobial agents, such as fluoride and chlorhexidine, into orthodontic adhesives by physical blending.^{2,3} However, these agents have some disadvantages, such as short-term release of antimicrobial agents and decreased mechanical properties.^{4,5}

Orthodontic composite bonding systems consist of a bonding primer and adhesive. The primer is used to provide a proper bond strength between the tooth and bracket.⁶ Because composite adhesives for orthodontic bonding are applied to the enamel surface after priming, an antimicrobial primer is beneficial for

reducing biofilms and demineralization at the interface between the bracket and the tooth surface.

Oral biofilms display enhanced pathogenicity when the biofilm bacteria are less susceptible to antimicrobial agents and mechanical cleansing.⁷ Therefore, one strategy to control enamel demineralization is to inhibit the formation of bacterial biofilms on teeth. Chlorhexidine (CHX) is widely used for chemical plaque control because it inhibits biofilm formation on tooth surfaces.⁸ Natural products derived from medicinal plants may also be useful as antimicrobial agents. Ursolic acid (UA) is a triterpenoid compound that has anticariogenic activity with relative nontoxicity.^{9,10}

The purposes of this study were to compare the antimicrobial properties of experimental primers containing CHX or UA with those of a commercial primer using both planktonic and biofilm models of *S. mutans* and to compare physical properties by analyzing shear bond strength and bond failure patterns.

MATERIALS AND METHODS

Transbond XT primer (TX; 3M Unitek, Monrovia, Calif) was used as a parent primer. According to the manufacturer, TX contains mainly 34%–55% bisphenol A diglycidyl ether dimethacrylate (bis-GMA); 45%–55% triethylene glycol dimethacrylate (TEGDMA); and small quantities (less than 1%) of triphenylantimony, 4-(dimethylamino)-benzeneethanol, comphoroquinone, and hydroquinone. For antimicrobial properties, 3 mg of CHX (Sigma-Aldrich, St Louis, Mo) or UA (Sigma-Aldrich) were incorporated into 1 ml of TX in a sealed bottle, named TX-CHX or TX-UA, respectively. TX-CHX or TX-UA was stirred for 12 hours at room temperature to obtain uniform mixtures.

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were determined according to the National Committee for Clinical Laboratory Standards.¹¹ Briefly, *S. mutans* UA159 was grown in brain heart infusion (BHI) (Becton Dickinson, Sparks, Md) broth at 37°C in a 5% CO₂ aerobic atmosphere at its midexponential phase (OD₆₀₀ = 0.5, approximately 6.5 × 10⁷ colony-forming units/mL). This was added to a polystyrene, 96-well plate (Costar 3595; Corning Inc, Corning, NY) to reach a final concentration of 1 × 10⁶ CFU/mL. To measure the MIC, primers were added to each well to final concentrations of 8, 16, 32, 64, 128, 256, 512, and 1024 µg/mL. Because bacterial growth in BHI broth containing 2% DMSO was no different from growth in BHI alone, 2% DMSO was used as a vehicle to load primers. A well containing ampicillin (final concentration, 100 µg/mL) was used as the positive control. Culture medium only and culture medium containing 2% DMSO were used as double-negative

controls. After incubating for 24 hours under appropriate conditions, the lowest primer concentration that inhibited visible growth was considered the MIC value. To determine the MBC value, 100 µL of bacterial culture from each well at the MIC value determined above was diluted 10- through 1000-fold and plated onto BHI agar plates for each bacterial strain. The agar plate was incubated at 37°C for 48 hours and the number of colonies was then counted. The concentration that killed 99.9% of the bacteria was considered the MBC value. All measurements were repeated three times on different days.

Growth assays were performed using a 96-well plate to analyze the effects of primers on the planktonic growth of *S. mutans*. Before assaying, primers were carefully applied to the bottoms of cell culture plates and light-cured for 20 seconds from the top and 20 seconds from the bottom using an Ortholux LED (3M Unitek, Orange, Calif) with a light intensity of approximately 1000 mW/cm². To compare growth rates, the BHI broth was inoculated with 1:100 dilutions of overnight cultures of *S. mutans* UA159 and the bacterial suspensions were incubated at 37°C in wells with primers. The optical density at 600 nm was recorded for 72 hours to monitor bacterial growth at routine time intervals using a microplate reader (Infinite F200 pro, Tecan GmbH, Grödig, Austria).

Because antimicrobial activity could gradually decrease by releasing antimicrobial agents from the primer into the oral cavity, plates were thermocycled in water to analyze the effects of water aging on antimicrobial activities and to simulate normal aging in the oral cavity. The thermocycling equipment (Kwanduk, Seoul, Korea) was set at 20-second dips to 5 seconds of transfer time. The immersion temperatures ranged from 5°C to 55°C. Five thousand cycles, corresponding to 6 months of aging,¹² were performed. After thermocycling, growth assays were performed again under the same environment. All assays were performed in triplicate and independently repeated three times.

Unstimulated whole saliva was collected by the expectorating of healthy volunteers as previously described.¹³ Subjects provided consent to the research protocol, which had been reviewed and approved by the Institutional Review Board. Saliva samples were centrifuged at 3500 × g for 10 minutes to remove any cellular debris and the resulting supernatant was used after filter-sterilization through a Stericup and Steritop (Millipore, Billerica, Mass).

The primers were carefully applied to plates as described above, then biofilm assays were performed using a 96-well plate. To assess biofilm formation, *S. mutans* UA159 was grown in a semidefined biofilm medium with 18 mM glucose and 2 mM sucrose as carbohydrate sources as previously described.¹³ Over-

night cultures of *S. mutans* were transferred to prewarmed BHI and grown at 37°C in a 5% CO₂ aerobic atmosphere until reaching the midexponential phase (OD₆₀₀ = 0.5). Cultures were then diluted 100-fold in prewarmed biofilm medium.

For saliva coating, each primer-conditioned well was conditioned with 60 µL of saliva, then biofilm assays were performed. The plates were incubated at 37°C for 2 hours with gentle shaking and then washed twice with phosphate-buffered saline (pH = 7.2). Immediately after air-drying for 30 minutes, the wells were inoculated with 150 µL of cell suspensions. After inoculation, all plates were incubated at 37°C in a 5% CO₂ atmosphere for 24 hours. The culture medium was then decanted, and the plates were washed twice with 150 µL of sterile distilled water to remove planktonic and loosely bound cells. Adherent bacteria were stained with 50 µL 0.1% crystal violet for 15 minutes. After rinsing twice with 150 µL of water, the bound dye was extracted from the stained cells using 200 µL of 99% ethanol. Biofilm formation was then quantified by measuring the absorbance of the solution at 600 nm using a spectrophotometer (Helios beta; Thermo Scientific, Madison, Wisc). The same experiments were repeated after thermocycling. All assays were performed in triplicate and independently repeated three times.

After 48 freshly extracted healthy bovine incisors were cleaned, bovine enamel pieces (8 × 3 mm) were prepared using a punching machine (Youngsoo, Seoul, Korea). The enamel pieces were embedded in an acrylic mold with the labial surfaces parallel to the mold base so that they would be parallel to the force during the shear bond test. The incisors were randomly assigned to one of three groups (16 incisors each). The bonding surface was then etched with 32% phosphoric acid gel for 20 seconds, rinsed with water for 30 seconds, and air-dried until the enamel had a faintly white appearance. Either TX, TX-CHX, or TX-UA was applied to the etched surface in a thin layer and air-dried. Then the maxillary incisor brackets (Artista series, Ortho-Direct, St Ann, Mo) were bonded on the labial surface of the incisors with Transbond XT composite adhesive (3M Unitek) and light cured for 20 seconds (10 seconds from the mesial and 10 seconds from the distal) using an Ortholux LED. After bonding, the specimens were thermocycled as described above. After thermocycling, shear bond strength was measured. A universal testing machine (Instron 4465, Canton, Mass) with a crosshead speed of 1 mm/minute was used. An occlusogingival load was applied to the brackets using a chisel-edged plunger, producing a shear load at the bracket-tooth interface. Maximum loads were recorded in kgf and then converted into MPa (nominal surface area, 9.91 mm²).

After the bond-strength test, the debonded surfaces were examined using a stereomicroscope (Nikon SMZ-U, Tokyo, Japan) at 10× magnification. The amount of adhesive left on the enamel surface was scored for each tooth using the adhesives remnant index (ARI).¹⁴ The ARI scale ranges from 0 to 3: 0 indicates no adhesive remaining on the tooth; 1, less than half of the enamel bonding site covered with adhesive; 2, more than half of the enamel bonding site covered with adhesive; and 3, enamel site covered entirely with adhesive.

Two-way ANOVA with Bonferroni correction was used to determine significant differences in biofilm formation with respect to primer type and thermocycling. Differences in shear bond strength were analyzed using the Kruskal-Wallis test. ARI scores were analyzed using Fisher's exact test. Values were considered significant when $P < .05$.

RESULTS

The MIC value of TX-CHX was 32 µg/mL, while those of TX and TX-UA were 1024 µg/mL. The MBC value of TX-CHX was 32 µg/mL, while those of TX and TX-UA were not detected (over 1024 µg/mL). This indicates that TX-CHX had more antimicrobial strength than did TX or TX-UA.

Growth assay results showed that application of TX-CHX to the plate bottom completely inhibited bacterial growth (Figure 1A,B). In addition, thermocycling did not significantly influence the growth inhibition effects of TX-CHX on *S. mutans* (Figure 1B).

TX-UA and TX increased the growth rate slightly compared with a no-primer control at an early stage, but the growth patterns were not significantly different among groups after 40 hours of exposure, irrespective of thermocycling (Figure 1A,B).

There were significant differences in biofilm development by *S. mutans* according to primer type (Table 1). TX, TX-UA, and TX-CHX significantly inhibited the biofilm formation of *S. mutans*, compared with the no-primer control, but inhibition patterns were different from each other. There were no significant differences in biofilm inhibition between TX and TX-UA, but TX-CHX completely inhibited biofilm formation of *S. mutans*, compared with TX and TX-UA. However, thermocycling did not significantly influence the effects of primers on the biofilm formation of *S. mutans*. This indicates that TX-CHX has stronger antimicrobial activities against *S. mutans* than does TX or TX-UA, irrespective of thermocycling.

Table 2 shows no significant differences in bond strength among primer groups. The ARI scores were not significantly different, meaning that no significant differences were found in the debond patterns among primer groups (Table 3). These results indicate that incorpora-

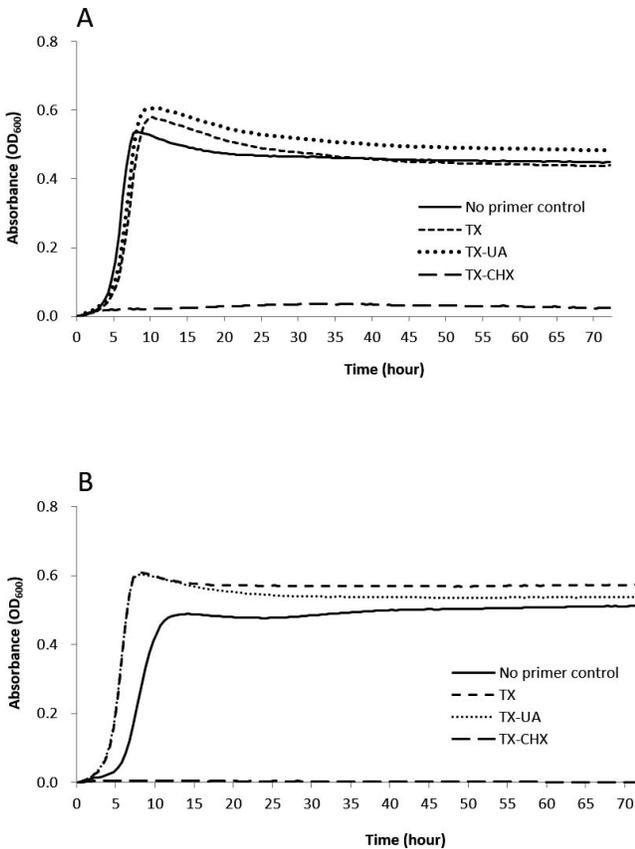


Figure 1. Time-related growth of *Streptococcus mutans* in primer-conditioned plates in the absence (A) or presence (B) of thermocycling. Bacterial growth was monitored using an Infinite F200 pro system for 72 hours.

tion of CHX or UA into the primer did not adversely affect the physical properties of the original primer.

DISCUSSION

An appropriate concentration of antimicrobial agents in the primer is important because insufficient amounts limit antimicrobial activity and excess amounts deteri-

orate the mechanical properties of materials. Results showed that 3 mg/mL of CHX or UA was the optimal concentration for antimicrobial activity, because more than 3 mg/mL of CHX or UA did not uniformly mix with TX without disturbing shear bond strength (data not shown).

The MIC and MBC tests are well-known in vitro sensitivity tests for antimicrobial agents against pathogenic bacteria, and low values indicate high potency for antimicrobial activity.¹¹ The MIC and MBC results showed that over 32 $\mu\text{g/mL}$ of TX-CHX completely inhibited growth of *S. mutans*, but TX and TX-UA inhibited growth of *S. mutans* at over 1024 $\mu\text{g/mL}$. In addition, TX-CHX killed 99.9% of *S. mutans* at over 32 $\mu\text{g/mL}$, but TX or TX-UA did not affect *S. mutans* at a detected level. The stronger antimicrobial property of TX-CHX originates from the stronger antimicrobial property of CHX compared with UA. In our preliminary study, we found that the MICs of CHX and UA against *S. mutans* UA159 were 1 $\mu\text{g/mL}$ and 8 $\mu\text{g/mL}$, respectively, while the MBCs were 1 $\mu\text{g/mL}$ and 16 $\mu\text{g/mL}$, respectively.

Unfortunately, incorporation of UA did not significantly enhance the antimicrobial activity of TX. This may be due to the interaction of UA with TX. Bis-GMA, TEGDMA, or other ingredients present in small amounts in TX can interact with UA, which may degrade or prevent the antimicrobial activity of UA against *S. mutans*.

Biofilm assays were performed in primer-conditioned plates after saliva coating, because the antimicrobial effect is significantly reduced by adsorption of salivary proteins on contact-killing dental materials.¹⁵ The results of biofilm assays showed that wells containing TX-CHX completely inhibited biofilm formation of *S. mutans*. Although application of TX or TX-UA inhibited biofilm formation of *S. mutans* compared with a no-primer control, the biofilm inhibitory effects of TX or TX-

Table 1. Comparisons of Biofilm Development by *Streptococcus mutans* With Respect to Primer Type and Thermocycling^a

Primers	Cycling	Biofilm Formation (Mean \pm SD)	Significance [†]
No primer control	No cycling	8.86 \pm 0.40	No cycling = Thermocycling Control > TX, TX-UA > TX-CHX*
	Thermocycling	8.85 \pm 0.23	
TX ^b	No cycling	7.56 \pm 0.34	
	Thermocycling	7.55 \pm 0.35	
TX-UA ^c	No cycling	7.29 \pm 0.26	
	Thermocycling	7.81 \pm 0.36	
TX-CHX ^d	No cycling	0.25 \pm 0.81	
	Thermocycling	0.00 \pm 0.00	

^a Biofilm assays were performed in primer-conditioned plates after saliva coating and biofilm formation was assayed on polystyrene microtiter plates after staining with crystal violet.

^b TX indicates Transbond XT primer, 3M, Monrovia, Calif.

^c TX-UA indicates Transbond XT primer containing 3 mg/mL of ursolic acid.

^d TX-CHX indicates Transbond XT primer containing 3 mg/mL of chlorhexidine.

[†] Two-way ANOVA was used to determine significant differences among groups using Bonferroni correction at a significance level of $\alpha < 0.05$;

* $P < .001$.

Table 2. Comparisons of Shear Bond Strengths (Mean ± Standard Deviation) With Respect to Primer Type

	TX ^a	TX-UA ^b	TX-CHX ^c	Significance*
Shear bond strength (MPa)	10.57 ± 1.81	12.09 ± 2.64	11.46 ± 2.30	Not significant
Range	7.77–14.51	8.84–17.62	8.02–15.36	

^a TX indicates Transbond XT primer, 3M, Monrovia, Calif.

^b TX-UA indicates Transbond XT primer containing 3 mg/mL of ursolic acid.

^c TX-CHX indicates Transbond XT primer containing 3 mg/mL of chlorhexidine.

* Differences in shear bond strength were analyzed using the Kruskal-Wallis test.

UA were significantly lower than those of TX-CHX (Table 1).

TX-CHX may have inhibited biofilm formation in two different ways. First, CHX, which immobilized into TX-CHX, may show antimicrobial effects against bacteria that contact CHX immobilized on the bottom of plates. At the beginning of biofilm formation, planktonic bacteria reversibly attached to the material surface,¹⁶ and CHX immobilized on the surface could have contacted and killed bacteria. Because the TX-CHX was covered with saliva, CHX may have diffused and penetrated through the saliva-coating to exert antibacterial effects that may not have been impeded by the saliva coating. Second, CHX molecules could have been eluted from TX-CHX into the environment. CHX is partly water soluble (less than 16 µg/mL was dissolved in water in our preliminary study), and eluted CHX significantly influences bacterial growth and consecutive biofilm formation. These hypotheses could be supported by the growth assay. Application of TX-CHX onto the bottom completely inhibited growth of *S. mutans* (Figure 1).

However, elution of CHX may decrease its long-term antimicrobial activity, because of limited quantities in the primer. To investigate whether the inhibitory effect is influenced by water aging, growth and biofilm assays were performed after thermocycling. Figure 1 and Table 1 reveals that TX-CHX still had a significant inhibitory effect on bacterial growth and biofilm

formation after thermocycling. There was no statistically significant difference in the inhibitory effect between noncycled and thermocycled plates. Because CHX is relatively hydrophobic (less than 16 µg/mL was dissolved in water in our preliminary study) and hydrophobic bis-GMA/TEGDMA primer mixes well with CHX, CHX may be stably immobilized in primer networks and released in a small quantity over time, thus providing durable antimicrobial capability. Considering that the MIC and MBC values of CHX against *S. mutans* UA159 were only 1 µg/mL, incorporation of 3 mg/mL CHX (3000 times that of MIC and MBC of CHX) may be enough to inhibit bacterial growth and biofilm formation even though a part of CHX was lost from the primers through elution.

Because bovine enamel has been used as a suitable alternative for human teeth in bonding tests,¹⁷ bovine incisors were used to analyze the bond strength and debond patterns. Table 2 demonstrates that TX-CHX or TX-UA has clinically relevant bond strength ranges compared with that of TX. In addition, there were no significant differences in bond failure patterns among the primers (Table 3). These results indicate that incorporation of 3 mg/mL CHX into orthodontic primers does not have an adverse effect on the physical properties of the parent primer.

The adhesive-enamel interface deteriorates due to cyclic fatigue exerted during mastication, which induces microgaps at the interface. Microgaps can harbor biofilms, and their acid production can cause demineralization at the bracket margin. A previous study has reported the presence of 10-µm-wide gaps at adhesive-enamel junctions around the bracket base, within which bacterial accumulation was consistently detected.¹⁸ The invading bacteria in marginal gaps directly contact primer rather than the enamel surface. Therefore, it is desirable that primers contain antimicrobial properties. In this regard, TX-CHX can have direct contact with new bacteria invading the adhesive-enamel margins and inhibit bacterial growth and biofilm formation at the interface.

Although *S. mutans* is a major contributor to biofilms in the oral cavity, *S. mutans* cells do not exist as a single species in the oral cavity. Because release of antimicrobial agents declines significantly within the first few days^{4,5} and thermocycling promotes antimicro-

Table 3. Frequency and Distribution of Adhesive Remnant Index (ARI) When Different Primers Were Used for Bonding Brackets

Primer Type	ARI Scores*				Significance [†]
	0	1	2	3	
TX ^a	5	6	1	4	Not significant
TX-UA ^b	4	5	0	6	
TX-CHX ^c	5	4	2	5	

^a TX indicates Transbond XT primer, 3M, Monrovia, Calif.

^b TX-UA indicates Transbond XT primer containing 3 mg/mL of ursolic acid.

^c TX-CHX indicates Transbond XT primer containing 3 mg/mL of chlorhexidine.

* 0 indicates no adhesive remaining on tooth; 1, less than half of enamel bonding site covered with adhesive; 2, more than half of enamel bonding site covered with adhesive; 3, enamel site covered entirely with adhesive.

[†] Fisher's exact test was performed to analyze differences in ARI scores between groups at a significance level of α = 0.05.

bial release from primers, 5000 thermocyclings for 7 days of water aging were performed in this study. Although 5000 cycles physically corresponds to 6 months of aging,¹² 7 days may be not enough to analyze long-term antimicrobial effects of primers. Further long-term in vivo studies are needed to investigate the physical and antimicrobial potentials of orthodontic primers.

CONCLUSIONS

- CHX incorporation provided stronger antimicrobial properties than did the control material, manifested in MIC, MBC, growth assay, and biofilm assays.
- Thermocycling for water aging did not significantly influence antimicrobial properties.
- UA-containing primer did not show significant antimicrobial activity.
- Incorporation of antimicrobial agents did not significantly influence the shear bond strength or bond-failure patterns.

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