Original Article

Effect of orthodontic bonding steps on the initial adhesion of mutans streptococci in the presence of saliva

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ABSTRACT

Objective: To test the hypothesis that orthodontic bonding has no effect on the initial adhesion of mutans streptococci (MS) in the presence of saliva.

Materials and Methods: Hydroxyapatite (HA) and orthodontic adhesive (AD) disks were prepared to a uniform size. HA disks were etched with 37% phosphoric acid (HE, etched group). Some of the HE disks were coated with Transbond XT primer and light cured (HP, primed group). Transbond Plus SEP was applied to a third set of HA disks, dried, and light cured (SEP, self-etching primer group). Adhesion assays were performed using two MS strains in the presence of fluid-phase or surface-adsorbed unstimulated whole saliva (UWS). The MS adhesion patterns were examined by scanning electron microscopy.

Results: MS adhesion was influenced by the bonding steps and the presence of UWS. UWS treatment decreased MS adhesion. However, surface-adsorbed UWS resulted in slightly less inhibition of MS adhesion than fluid-phase UWS. MS adhesion was significantly greater for HE than for the other groups. There were interaction effects between the UWS treatment and surface groups. MS adhesion to HP and AD was significantly diminished in the presence of surface-adsorbed or fluid-phase UWS compared with adhesion to HA, HE, or SEP.

Conclusion: The hypothesis is rejected. Our results suggest that MS adhesion is significantly influenced by the bonding procedure used, and the application of conventional primers for the bracket bonding can inhibit MS adhesion to tooth surfaces in the presence of UWS. (*Angle Orthod.* 2011;81:326–333.)

KEY WORDS: Orthodontic bonding; Bacterial adhesion; Mutans streptococci; Saliva

INTRODUCTION

Fixed orthodontic appliances contribute to the adhesion of oral bacteria due to their complex design, which prevents proper cleaning around orthodontic brackets and may result in enamel demineralization. The enamel demineralization is caused by organic acids produced mainly by mutans streptococci (MS).[1] MS adhesion to tooth surfaces is the first step in the formation of biofilms by this organism, which plays a key role in the development of enamel demineralization. Of these species, *Streptococcus mutans* and *Streptococcus sobrinus* are most frequently isolated from the human oral cavity and have been implicated as the primary agents of human dental caries.[2] The placement of fixed orthodontic appliances leads to an increase in the level of MS within dental plaque, while MS levels return to normal after removal of the appliance.[3,4]

Saliva plays significant roles in the adhesion of MS to intraoral surfaces in two different ways. Saliva can mediate the aggregation of MS by interaction with the cell surface adhesin (antigen I/II family) of MS in the fluid phase, or saliva can provide sites for initial adherence of the organisms to oral surfaces in the surface phase.[5] As a result, saliva can facilitate

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bacterial clearance from the oral cavity through aggregation and promote adhesion of MS by serving as specific receptors.

The orthodontic bonding procedure includes a number of steps, such as etching, priming, and application of orthodontic adhesive. In addition, acid-etch primers were recently introduced to eliminate the need for separate etching, rinsing, and drying steps. Many studies have been conducted to evaluate the performance of orthodontic bonding systems for more successful bonding. However, few studies have investigated changes in MS adhesion after surface changes by bracket bonding steps. Changes in surface morphology followed by the bonding procedure not only may change mechanical bond strength but also may influence MS adhesion. The purpose of this study was to analyze the effects of orthodontic bonding steps on MS adhesion using hydroxyapatite disks in the presence of saliva.

MATERIALS AND METHODS

Hydroxyapatite disks were prepared to a uniform size (5.0-mm diameter and 2.0-mm thickness) by the sintering of regent-grade $Ca(PO_4)_3OH$ powder (Sigma, St Louis, Mo) as previously described.[6] The disks were divided into four groups according to surface treatment type: no surface treatment control (HA), acid-etched (HE), primed using a conventional primer after acid-etching (HP), and primed using a self-etching primer (SEP).

In the HE group, hydroxyapatite (HA) was etched with 37% phosphoric acid gel (Etchant, Bisco, Schaumburg, Ill) for 20 seconds and rinsed with deionized water. In the HP group, Transbond XT primer (3M/Unitek, Monrovia, Calif) was applied to HE in a thin film and light cured for 30 seconds with Ortholux LED (3M/Unitek). In the SEP group, Transbond Plus SEP (3M/Unitek) was applied and rubbed on HA, and the surfaces were light cured for 30 seconds with Ortholux LED. Transbond XT composite adhesive (3M/Unitek) disks were prepared to the same uniform size using Teflon templates in the adhesive (AD) group as previously described.[6]

Unstimulated whole saliva (UWS) was collected from healthy volunteers as previously described.[7] Each saliva sample was centrifuged at 3500*g* for 10 minutes to remove any cellular debris, and the resulting supernatant was used after filter sterilization through a Stericup & Streitop (Millipore, Billerica, Mass).

S. mutans UA159 and *S. sobrinus* ATCC 33478 were maintained in brain-heart infusion (BHI) medium. Cells from exponential-phase cultures ($OD_{600} = 0.5$) were washed two times with phosphate-buffered saline (PBS; pH = 7.2) and resuspended to an $OD_{600} = 0.5$ (approximately 6.5×10^7 colony-forming unit [CFU] per milliliter). Each disk was placed in polystyrene 96-well cell culture clusters (Corning Inc, Corning, NY). Adhesion assays were performed in three different ways: surface-adsorbed UWS (S-UWS), fluid-phase UWS (F-UWS), or no saliva treatment (control). For the experiments with S-UWS, each disk was conditioned with 100 μL of UWS in the well at 37°C for 2 hours with gentle shaking, followed by three washes with PBS. After air drying for 30 minutes, 150 μL of the cell suspensions ($OD_{600} = 0.5$) was added into the wells. For experiments with F-UWS, 150 μL of the cell suspension was inoculated into the wells containing each specimen concurrently with 15 μL of UWS. In the case of controls, 150 μL of the cell suspension was incubated with each specimen without any saliva treatment. After 3 hours of incubation, the cell suspensions were decanted and the specimens were washed twice with 200 μL of sterile PBS to remove loosely bound cells. Each specimen was then transferred to a cornical tube containing 3 mL of sterile PBS. The adherent bacteria were then detached by sonication using four 30-second pulses at 20 W with three 30-second intermittent coolings in an ice box. The cell suspensions were serially diluted, plated on BHI agar, and incubated at 37°C for 2 days before colonies were counted. Colony counts were expressed as a CFU per unit area of the specimens (cm^2). All assays were performed in duplicate and repeated five times.

To examine the adhesion patterns of MS to various surfaces by scanning electron microscopy (SEM), MS adhesion assays were performed as described above, and the adhesion pattern was observed with a magnification set at 3000× using S-4700 microscopy (Hitachi, Tokyo, Japan).

The arithmetic mean of surface roughness (SR) of each specimen was analyzed within a sampling area ($245 \times 245 \times 60$ μm) using confocal laser scanning microscopy (Axiovert 200M, Carl Zeiss, Thornwood, NY). Each SR reading was performed three times on three different areas for each of the three specimens.

Factorial analysis of variance was used to analyze the amount of adhesion with respect to surface group and UWS treatment. Multiple comparisons were performed to analyze differences between groups by *t*-tests using the Bonferroni correction. SR was analyzed using the Kruskal-Wallis test, and the Mann-Whitney *U*-test was performed to compare differences between groups. Values were considered significant when $P < .05$.

RESULTS

The adhesion of *S. mutans* was significantly influenced by the surface type and the presence of

Table 1. Adhesion of *Streptococcus mutans* to Various Surfaces in the Presence of Unstimulated Whole Saliva[a]

	Surface Treatment ($\times 10^6$ CFU/cm^2)					
Saliva Treatment	HA Mean (SD)	HE Mean (SD)	HP Mean (SD)	SEP Mean (SD)	AD Mean (SD)	Significance*
No treatment	1.27 (0.42)	2.39 (0.95)	2.58 (1.23)	1.79 (0.84)	2.02 (0.90)	HA, HP, AD, SEP < HE
Surface adsorbed	0.49 (0.09)	1.57 (0.52)	0.29 (0.20)	1.06 (0.37)	0.28 (0.19)	No saliva treatment >
Fluid phase	0.53 (0.17)	0.84 (0.31)	0.32 (0.21)	0.71 (0.46)	0.31 (0.15)	surface adsorbed > fluid phase

[a] The amounts of biofilms were expressed as a colony-forming unit per unit area ($\times 10^6$ CFU/cm^2). HA indicates hydroxyapatite disk with no surface treatment; HE, hydroxyapatite disk with acid-etched treatment; HP, HE with primer (Transbond XT primer, 3M/Unitek, Monrovia, Calif) treatment; SEP, hydroxyapatite disk with self-etching primer (Transbond Plus SEP, 3M/Unitek) treatment; and AD, orthodontic adhesive disk (Transbond XT, 3M/Unitek).

* Multiple comparisons were performed by *t*-tests using the Bonferroni correction at a significance level of $P = .05$.

UWS (Table 1). Multiple comparisons demonstrated that adhesion of *S. mutans* was higher in the HE than in other experimental groups (HE > HA, HP, AD, and SEP). UWS treatment considerably inhibited the adhesion of *S. mutans* to various surfaces, but there were significant differences between S-UWS and F-UWS (no saliva > surface adsorbed > fluid phase). There were significant interactions between the surface group and UWS treatment. A decrease in the adhesion of *S. mutans* with UWS treatment was more evident in HP and AD than in the other groups, particularly in the presence of S-UWS.

Adhesion patterns of *S. sobrinus* were similar to those of *S. mutans*, although adhesion levels of *S. sobrinus* were generally lower than those of *S. mutans* (Table 2) Adhesion of *S. sobrinus* in the HE group was highest, while there were no significant differences in the adhesion of *S. sobrinus* among the other four groups (HE > HA, HP, AD, and SEP). Both S-UWS and F-UWS significantly decreased adhesion of *S. sobrinus* to various surfaces. However, S-UWS resulted in slightly less inhibition of bacterial adhesion than F-UWS (no saliva > surface adsorbed > fluid phase). Like *S. mutans*, the decrease in adhesion of *S. sobrinus* was more evident in HP and AD than in the other groups in the presence of S-UWS.

MS adhesion patterns examined by SEM were consistent with the quantitative adhesion data. Adhesion patterns of *S. mutans* were more clustered than those of *S. sobrinus* (Figures 1 and 2). Adherent cell clusters (Figure 1; *S. mutans*) and longer chains (Figure 2; *S. sobrinus*) were shown on the surfaces in the absence of UWS, while the scattered cells (Figure 1; *S. mutans*) and short chains (Figure 2; *S. sobrinus*) were observed on surfaces in the presence of UWS. Adherent cell clusters and cell aggregates were more abundant in the HE, HP, and AD groups than in the HA and SEP groups in the absence of UWS treatment. However, the differences in MS adhesion patterns between the groups were less evident in the presence of UWS, particularly in the presence of F-UWS.

There were significant differences in SR among the experimental groups (Table 3). HE showed the roughest surface, and AD showed the smoothest surface. There was no significant difference in SR among HA, HE, and SEP.

DISCUSSION

This study showed that UWS treatment significantly influenced MS adhesion. Although both UWS phases decreased MS adhesion, there were some differences between S-UWS and F-UWS. S-UWS inhibited MS adhesion less than F-UWS did (Tables 1 and 2). This may be due to the different roles of UWS in MS adhesion according to its phase. S-UWS may act as a barrier of MS adhesion by decreasing the surface free energy of the underlying materials.[8] As a result, such surface modification by saliva coating may reduce the

Table 2. Adhesion of *Streptococcus sobrinus* to Various Surfaces in the Presence of Unstimulated Whole Saliva[a]

	Surface Treatment ($\times 10^6$ CFU/cm^2)					
Saliva Treatment	HA Mean (SD)	HE Mean (SD)	HP Mean (SD)	SEP Mean (SD)	AD Mean (SD)	Significance*
No treatment	0.55 (0.18)	1.24 (0.51)	1.53 (0.87)	0.24 (0.11)	0.89 (0.51)	HA, HP, AD, SEP < HE
Surface adsorbed	0.44 (0.15)	0.61 (0.19)	0.10 (0.06)	0.85 (0.22)	0.12 (0.11)	No saliva treatment >
Fluid phase	0.32 (0.12)	0.45 (0.33)	0.22 (0.16)	0.30 (0.18)	0.21 (0.15)	surface adsorbed > fluid phase

[a] The amounts of biofilms were expressed as a colony-forming unit ($\times 10^6$ CFU). HA indicates hydroxyapatite disk with no surface treatment; HE, hydroxyapatite disk with acid-etched treatment; HP, HE with primer (Transbond XT primer, 3M/Unitek, Monrovia, Calif) treatment; SEP, hydroxyapatite disk with self-etching primer (Transbond Plus SEP, 3M/Unitek) treatment; and AD, orthodontic adhesive disk (Transbond XT, 3M/Unitek).

* Multiple comparisons were performed by *t*-tests using the Bonferroni correction at a significance level of $P = .05$.

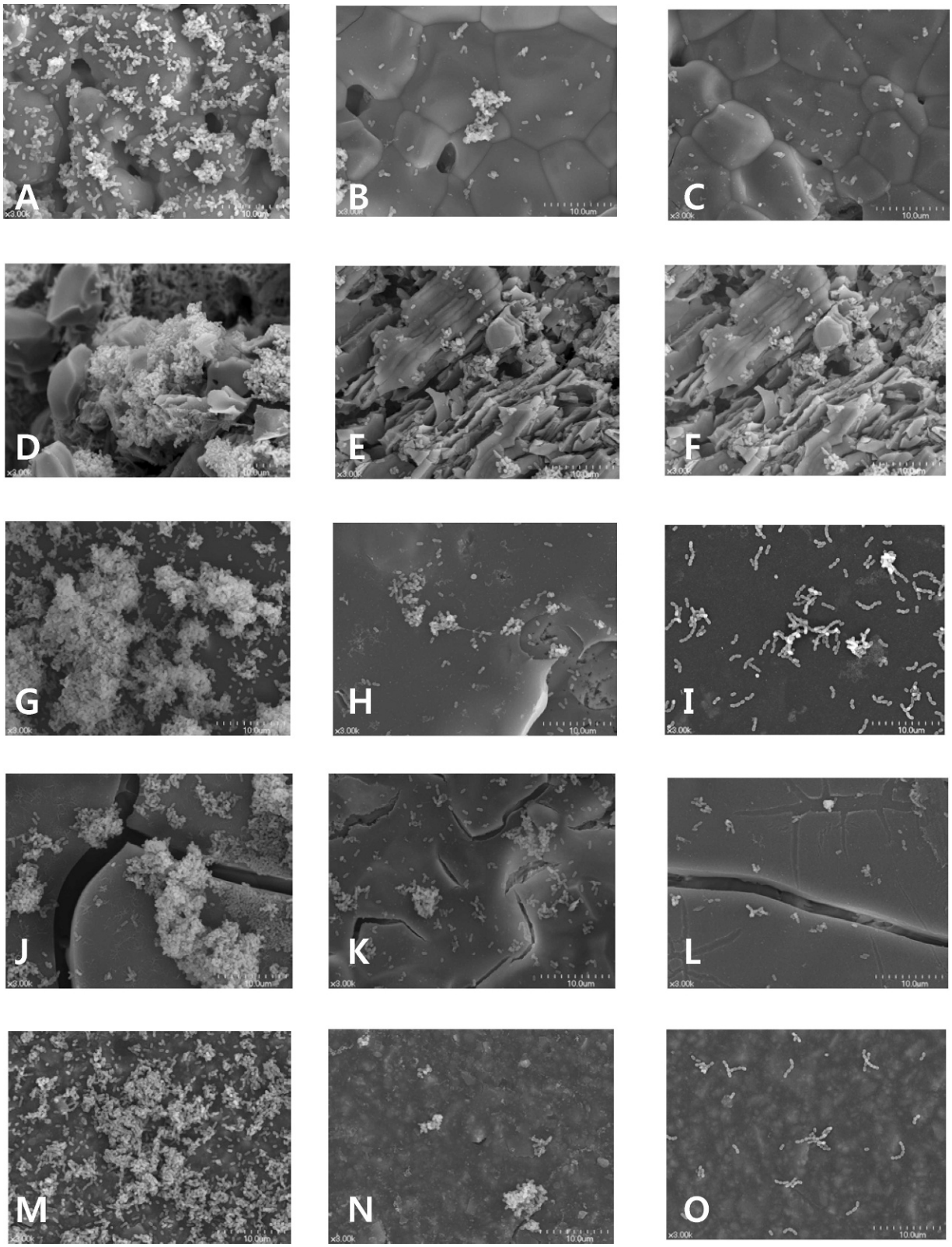

Figure 1. Scanning electron microscopic images of the adhesion of *Streptococcus mutans* to various surfaces. (A) Hydroxyapatite disk without surface treatment (HA) and saliva treatment. (B) HW with surface-adsorbed unstimulated whole saliva (S-UWS). (C) HA with fluid-phase UWS (F-UWS). (D) HA with an etched surface (HE) and no saliva treatment. (E) HE with S-UWS. (F) HE with F-UWS. (G) HE with a primed surface (HP) and no saliva treatment. (H) HP with S-UWS. (I) HP with F-UWS. (J) HA with self-etching primer treatment (SEP) and no saliva treatment. (K) SEP with S-UWS. (L) SEP with F-UWS. (M) Orthodontic adhesive (AD) without saliva treatment. (N) AD with S-UWS. (O) AD with F-UWS.

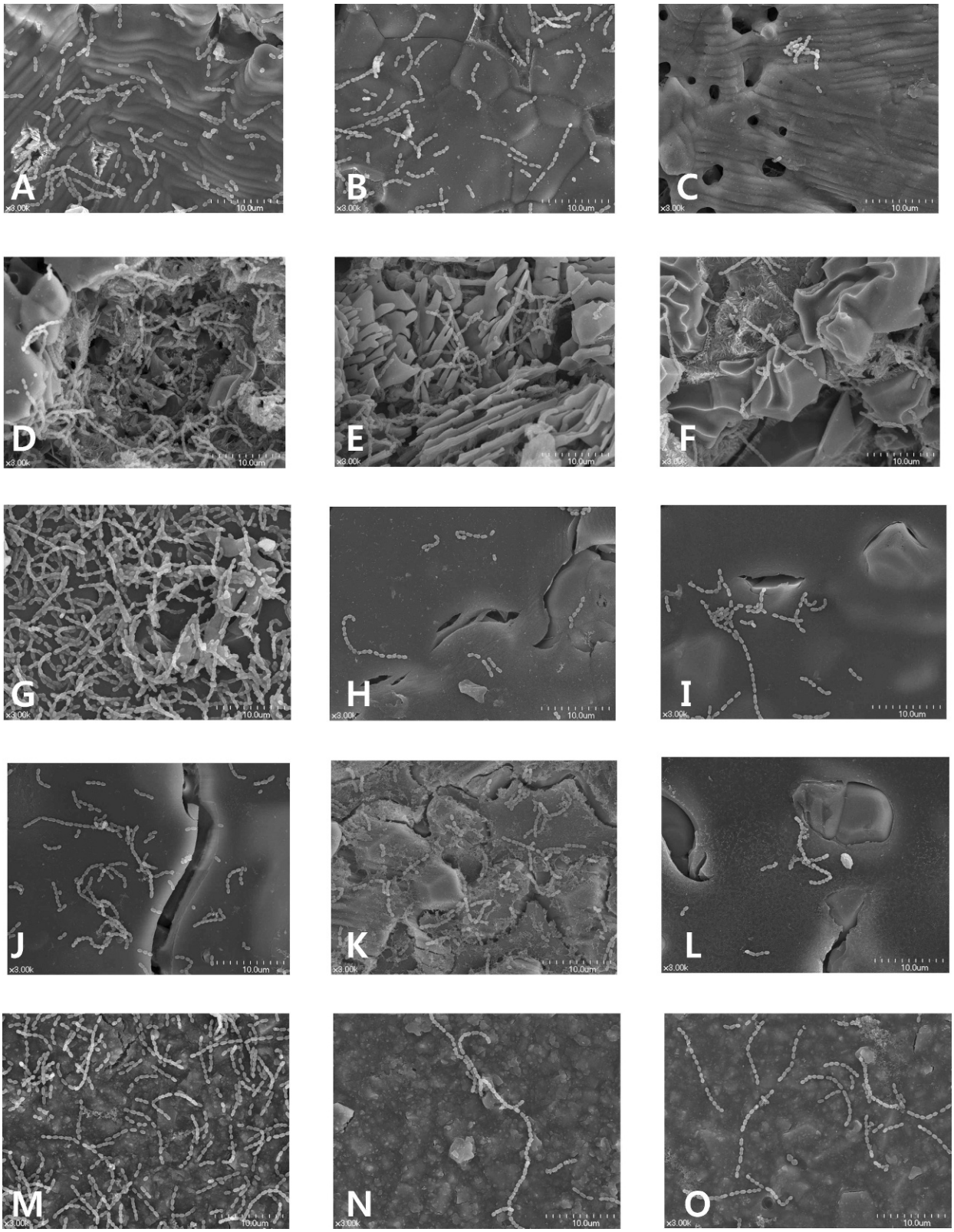

Figure 2. Scanning electron microscopic images of the adhesion of *Streptococcus sobrinus* to various surfaces. (A) HE without surface treatment (HA) and saliva treatment. (B) HW with surface-adsorbed unstimulated whole saliva (S-UWS). (C) HA with fluid-phase unstimulated whole saliva (F-UWS). (D) HA with an etched surface (HE) and no saliva treatment. (E) HE with S-UWS. (F) HE with F-UWS. (G) HE with a primed surface (HP) and no saliva treatment. (H) HP with S-UWS. (I) HP with F-UWS. (J) HA with self-etching primer treatment (SEP) and no saliva treatment. (K) SEP with S-UWS. (L) SEP with F-UWS. (M) Orthodontic adhesive (AD) without saliva treatment. (N) AD with S-UWS. (O) AD with F-UWS.

Table 3. Surface Roughness (μm) of Various Surfaces Used in This Study[a]

	HA Mean (SD)	HE Mean (SD)	HP Mean (SD)	SEP Mean (SD)	AD Mean (SD)	Significance*
Surface roughness	2.63 (0.27)	9.20 (3.58)	3.38 (0.89)	1.88 (0.54)	0.78 (0.19)	AD < HA, HP < HE SEP < HE

[a] HA indicates hydroxyapatite disk, no surface treatment; HE, hydroxyapatite disk, acid-etched treatment; HP, HE primer (Transbond XT primer, 3M/Unitek, Monrovia, Calif) treatment; SEP, hydroxyapatite disk with self-etching primer (Transbond Plus SEP, 3M/Unitek) treatment; and AD, orthodontic adhesive disk (Transbond XT, 3M/Unitek).

* Kruskal-Wallis test was used to analyze the difference in age and treatment times between the three groups at a significance level of $P = .05$.

strength of bacterial adhesion to the substratum, resulting in decreases in the amounts of adherent bacteria. F-UWS may inhibit MS adhesion in a different way, because the cells have an opportunity to directly adhere to the surfaces without the interference of saliva coating before coating completes. Generally, the inhibition patterns by F-UWS were similar when more than 3.3% F-UWS of the total volume (5 μL in this study) was added to the adhesion media. This may be due to the fact that bacterial aggregation induced by the interaction of F-UWS with MS may facilitate bacterial clearance from surfaces during washing, which may uniformly reduce MS adhesion to the underlying surfaces (Tables 1 and 2). The difference effects between S-UWS and F-UWS can be partly explained by the fact that S-UWS provides receptors for bacterial binding,[5,7] which may increase MS adhesion. Inhibition effects of both UWS treatments on MS adhesion were less significant on the rougher surfaces (HP and SEP) than on the smoother surfaces (HA, HP, and AD). This may be explained by the fact that rough surfaces provide microstructures for preventing the dislodgement of bacteria.

There were also substantial differences in MS adhesion according to surface type. MS adhered to HE more than to the other groups. The other four groups also showed some differences, but these differences were not statistically significant (Tables 1 and 2). The difference in MS adhesion can be due to the different surface characteristics of each surface type.

The primary effect of etching is to increase the surface area and thereby change the surface from a low-energy surface to a high-energy surface.[9,10] This study showed marked surface changes, with a significant increase in SR after acid etching (Table 3). Although the well-known honeycombed appearance was not observed, irregular microridges and microgrooves were shown in the HE group (Figures 1E and 2E). Rough surfaces provide opportunities for bacterial adhesion by increasing the surface area and providing suitable niches.[8,11] The present study also showed a significant increase in MS adhesion after acid etching, regardless of UWS treatment.

Priming is the second step of the conventional orthodontic bonding procedure and is necessary to provide micromechanical retention by penetration of the primer component into the irregular surface and to improve resistance to microleakage.[12] This study showed that rough surfaces left after acid etching smoothed and that the SR of the etched surface was returned to the original level (Table 3) due to the addition of a primer layer over the etched surface. However, MS adhesion to HP was not diminished in the absence of UWS treatment despite a decrease in roughness (Tables 1 and 2). This can be explained by the following. First, the primer layer may inhibit the dislodging of MS in the absence of saliva. The primer mainly consists of triethylene glycon dimethacrylate (TEGDMA) and bisphenol-A glycidyl methacrylate (GMA); (informed from the manufacturer), which were hydrophobic.[13] An increased surface hydrophobicity makes it easy for the bacteria to remain attached by relatively strong forces that mediate adhesion to the HP surfaces. Second, the unreacted primer components on the surfaces may increase MS adhesion. Previous studies have reported that resin composite monomers significantly influence cellular functions and virulence by penetrating membranes and cross-reacting with intracellular molecules.[14,15] We also found that the main components of the primer, TEGDMA and Bis-GMA increased MS adhesion by about 10%–20%, even when a lower concentration (1:100,000 to 1:1,000,000) was present in the adhesion media (data not shown).

However, UWS treatment decreased MS adhesion more in HP than in HA or HE, particularly in the presence of S-UWS (Tables 1 and 2). The significant decrease in MS adhesion may be associated with the changes in surface characteristics and/or surface hydrophobicity on the surfaces by either S-UWS or F-UWS. Recently, salivary esterases have been reported to contribute to the breakdown of resin polymeric matrix and its constitutive monomers such as TEDGMA and Bis-GMA.[16] Unknown salivary components may prevent MS adhesion to HP by interaction with active primer components that promote MS adhesion.

MS adhesion patterns to AD were similar to those to HP, although MS adhesion levels to AD were lower than those to HP. This may be due to the similar compositions of the primer and adhesive. Transbond

XT adhesive consists of 70% inorganic fillers, 10%–20% Bis-GMA, 5%–10% bisphenol-A ethoxylate dimethacrylate, and less than 2% silane (information obtained from the manufacturer). Except for inorganic fillers, the other components are hydrophobic, which may enhance MS adhesion in the absence of UWS treatment (Tables 1 and 2). In the presence of either S-UWS or F-UWS, MS adhesion to AD was significantly diminished compared with HA or HE, which may be explained by the same reasons as for HP.

Recently, self-etching primers have become more popular in clinical orthodontics because they eliminate the washing and drying stages, which allows the clinician to minimize chair time and technique sensitivity. However, this study showed different effects on MS adhesion for conventional and self-etching primers (Tables 1 and 2). MS adhered to HP more than to SEP in the absence of UWS treatment, while MS adhered more to SEP than to HP after UWS treatment. In particular, the difference between HP and SEP was more evident in the presence of S-UWS, and adhesion of *S. sobrinus* to SEP was highest among the five surfaces in the presence of S-UWS (Figure 2K). This may be due to differences in composition between conventional and self-etching primers. Acid or another component that remained on SEP may be one of the reasons for the different MS adhesion levels between them. Further studies will be necessary to investigate the role of self-etching primer in bacterial adhesion in the presence of saliva.

MS adhesion to enamel surfaces is considered to be an important step in the development of enamel demineralization around orthodontic appliances. Our results indicate that the use of a conventional primer and orthodontic adhesive on tooth surfaces can inhibit MS adhesion to enamel surfaces in the presence of UWS. However, the clinical methods used to prepare orthodontic adhesive result in a much rougher surface than the methods used to prepare the adhesive surfaces (between glass slab and Teflon) in this study. In addition, a recent study reported the presence of 10-μm-wide gaps at the adhesive-enamel junction, within which bacterial accumulation was consistently detected.[17] These issues suggest that bonding adhesives around brackets should be carefully removed, even though MS adhesion was lower for AD than HA, HE, and SEP. Periodic coating using a conventional primer may help protect tooth surfaces against MS adhesion in the oral cavity.

CONCLUSIONS

- S-UWS decreased MS adhesion to surfaces more than F-UWS did. MS adhered more to HE than to other surfaces.
- Among the experimental groups, MS adhesion to HP and AD was lowest in the presence of both UWS phases.
- Periodic primer coating can protect enamel adjacent to brackets against MS adhesion in the oral cavity.

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