[Title page]

Initial *In vitro* bacterial adhesion on dental restorative materials

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Abstract

Objectives. The purpose of this study was to evaluate initial bacterial adhesion on several restorative materials with similar roughness.

Materials and methods. Sixty cylindrical slabs were prepared from four restorative materials: zirconia (Zr), alumina-toughened zirconia (Al-Zr), type III gold alloy (Au), and cp-titanium (Ti). All the materials were polished until mirror-like shine was achieved. The average surface roughness and topography were determined by atomic force microscopy. Contact angles were measured to calculate surface free energy by the sessile drop technique. After the formation of a salivary pellicle, *S. sanguinis, S. gordonii,* and *S. oralis* were inoculated onto the specimens and incubated for 4 h. Quantification of the adherent bacteria was performed by crystal violet staining technique and resazurin reduction assay. One-way ANOVA and Tukey's post hoc test were adopted for statistical analysis. The level of significance was 0.05.

Results. The R_a values determined with atomic force microscopy for all specimens were lower than 5 nm. Surface free energy increased in the order of Al-Zr, Zr, Ti, and Au. Differences were significant between the investigated materials in both crystal violet absorbance and fluorescence

intensities. Gold alloy showed the highest values for all bacterial strains ($P < 0.05$).

Conclusions. Zirconia and titanium may be more suitable than gold alloy as an abutment material with respect to the initial bacterial adhesion and subsequent advance of periimplantitis.

Introduction

The use of endosseous dental implants has become a reliable treatment option for restoring edentulous jaws (1). For long term success of implant-supported restoration, the prevention of marginal bone loss around the inserted implant is required. Aespecially, plaque management on the surface of implants has become an important issue since plaque accumulation on implant surfaces may cause inflammatory reactions leading to peri-implant bone loss (2, 3).

Biofilm formation on the implant surface is similar to that of teeth (4). Once the substratum is exposed to the saliva, acellular proteinaceous film called the salivary pellicle is formed. Bacteria do not adhere to the surfaces directly, but always adhere to the pellicle. Among over several hundred species, the *Streptococci* are the dominant pioneer species (5). These early colonizers prepare a favorable condition for the following colonizers and provide a binding site preferred by the putative periodontal pathogens (*Fusobacterium, Porphyromonas* species) (6). Reducing the number of initially adhering bacteria is an important characteristic of the implant surface. Various factors affect bacterial adhesion, such as surface free energy (SFE), hydrophilicity, surface chemistry, surface charge, roughness, and the presence of proteins (7). Many studies were

carried out on bacterial adhesion on biomaterials with various roughnesses and physicochemical properties. Based on the results of these studies, it was determined that biofilm formation is facilitated by increased surface roughness and SFE (8).

Titanium, zirconia, and gold can be considered as materials of choice for transgingival abutment. Among them, the application of zirconia as an abutment material has increased due to the recent trend of metal-free dentistry. Even though the increased cost of gold alloy has resulted in the use of cheaper materials, UCLA gold abutment has maintained its position in the market due to its abilities to resolve several compromising issues (9). Accrued studies so far have focused on the adherence of oral bacteria on the surface of titanium or zirconia (10, 11). Several modified versions of these studies have attempted to perform similar experiments on surface-modified titanium (12, 13). When it comes to gold alloy, however, there is no plausible data involving the relative adherence of oral bacteria on gold, titanium, and zirconia under controlled surface conditions.

The objective of this study was to investigate the adhesion of three initial colonizers, *S. sanguinis, S. oralis,* and *S. gordonii*, to titanium, gold, and two kinds of dental ceramic restoratives with similar surface roughness values in the presence of an acquired pellicle. The null hypothesis

underlining this study was that there were no differences among the investigated transgingival abutment materials in the adhesion of the initial colonizers.

Materials and methods

1. Specimens

Sixty cylindrical slabs (each with diameter of 5 mm, height of 1.1 mm, surface area of 19.63 mm^2) were prepared from zirconia (Zr), alumina toughened zirconia (Al-Zr), type III gold alloy (Au), and titanium (Ti). The surfaces were mechanically polished by wet grinding with abrasive paper (400-4000 grit successively, Buehler) followed by a felt disc in conjunction with 1 ㎛ diamond slurry spray. Through this method, one side of the specimens was polished to a mirror-like shine. After polishing, each specimen was cleaned with acetone, 70% (v/v) ethanol, and then finally rinsed with sterile distilled water and dried.

2. Determination of surface roughness and surface free energy

Several surface roughness parameters (R_a, R_q, R_z, R_{pv}) and topography were determined at three specimens of each material using atomic force

microscopy (XE-100, Park Systems, Korea) in a contact mode of 5 × 5 μm^2. For the characterization of surface energy, the sessile drop method was used. Contact angles were carried out using a video contact angle analyzer (General type Phoenix 150, SEO, Korea). Three samples of each group were used to determine the contact angle of deionized water and formaldehyde. One drop of probe liquid was deposited on the surface of the specimen. Contact angle (degree) was calculated with the software provided with the equipment. The total SFE was calculated using the Owens-Wendt method (14).

3. Saliva preparation

Unstimulated human whole saliva was collected and frozen immediately after collection. The samples were defrosted and sterilized immediately before the experiments using single-use syringe filters (0.45 and 0.22 μm, successively).

4. Bacteria preparation

The strains of *S. oralis, S. gordonii,* and *S. sanguinis* were grown in sterile trypticase soy yeast extract medium [30 g tryptic soy broth (BD diagnostics 211825) + 3 g yeast extract (sigma Y1625)] (Table 1). By

continuously controlling the optical density of the cell suspensions, a growth curve was obtained, and cells in the exponential phase were used for the experiments.

The optical density of bacterial suspension was adjusted to 0.004 at 550 nm (ND-1000, Thermo Scientific, Massachusetts, USA), which corresponds to a microbial concentration of 4.9 X 10^6 cells/ml.

5. Crystal violet staining

Thirty specimens were prepared for each material, using 10 specimens per strain. The specimens were incubated with sterilized saliva for the formation of an acquired salivary pellicle. After an incubation time of 2 hr at 37℃, the specimens were carefully rinsed with PBS and incubated with 1 ml of bacterial suspension at 37℃. After incubation for 4 hr, the specimens were gently rinsed twice with PBS to remove non-adhered bacteria, and the adhered bacteria were stained with 0.3% crystal violet (CV) solution for 10 minutes. The specimens were then gently washed twice with PBS, and 400 ㎕ destaining solution (80% EtOH + 20% acetone) was applied. After 10 minutes, 200 ㎕ solution of each well was transferred to a 96-well microplate, and the absorbance of CV was

measured using an ELISA Reader (Bio-Rad model 550, California, USA) at 550 nm.

6. Resazurin reduction assay

Just like with CV staining, 30 specimens were prepared for each material, using 10 specimens per strain. A resazurin reduction assay was also performed to determine the total quantity of adhering bacteria, as described in previous investigations (12). Thirty specimens of each material were transferred to 96-well cell clusters, and the relative fluorescence intensity of each specimen prior to the adhesion assay was determined using an automated multi-detection reader (Fluostar Optima, BMG Labtech, Offenburg, Germany). Subsequently, the specimens were incubated with sterilized saliva to simulate an acquired salivary pellicle. After an incubation time of 2 h at 37℃, the specimens were carefully rinsed with PBS and incubated with 1 ml of bacterial suspension and 15 $\mu\ell$ resazurin (Resazurin, Sigma-Aldrich, St. Louis, MO, USA) at 37℃. After incubation for 2.5 hr, the specimens were gently rinsed twice with PBS to remove non-adhered bacteria, and the relative fluorescence intensities after adhesion were measured.

7. Preparing for scanning electron microscopy

To observe the morphology of bacterial adhesion, the specimens were prepared for scanning electron microscopy. The specimens were fixed in 2% glutaraldehyde for 24 hr at room temperature, washed three times with phosphate buffer solution (pH 7.4), and dehydrated through a series of graded ethanol solutions (20%, 40%, 60%, 80%, and 100%). The samples were subsequently vacuum dried, sputter-coated with Au, and observed using a field emission scanning electron microscope (S-4700, HITACHI, Tokyo, Japan) with an accelerating voltage of 15 kV.

8. Statistics

Statistical analysis was carried out with PASW Statistics Ver. 18 (SPSS Inc., Chicago, IL, USA). One-way ANOVA was adopted followed by the Tukey's post hoc test. The probability of type I error less than or equal to 0.05 was considered statistically significant.

Results

1. Surface roughness

The average surface roughness of each material is summarized in Table 2. All specimens yielded a R_a lower than 5 nm. Figure 1 shows the topography of each polished surface.

2. Contact angle and surface free energy

The results of the sessile drop measurements on the test materials are shown in Table 3. The total SFE values (γs) were 37.88, 33.89, 59.92, and 47.67 mJ/m^2 for Zr, Al-Zr, Au, and Ti, respectively.

3. Crystal violet assay

One-way ANOVA revealed significant differences in CV absorbance among the investigated materials (Fig. 2). The post hoc analysis showed that, for all bacterial strains, gold alloy revealed significantly higher values than the other materials ($P < 0.001$). Titanium demonstrated a higher value than Zr and Al-Zr for *S. gordonii*. In most specimens, there was no difference in adhesion according to the bacterial strain except for Zr, in which specimen *S. sanguinis* exhibited significantly lower values than *S. oralis*.

4. Resazurin reduction assay

Significant differences were found in fluorescence intensities among the investigated materials (Fig. 3). The intensities of gold alloy were found to be the highest for all bacterial strains ($P < 0.001$), and these results are similar to those of CV absorbance.

5. Scanning electron microscopy

Adhesion on the test substrates was confirmed through scanning electron micrographs of the initial biofilm. Three bacterial strains exhibited similar adhesion patterns and *S. oralis* on 4 materials are represented in Fig. 4. A monolayer of characteristic streptococcal chains evenly adhered to the substrate.

Discussion

On the base of this study, gold may be carefully used as an abutment material because of the easiness of adhesion on its polished surface. The bacterial adhesion is important in the selection of the material used in the transgingival portion, along with other physical properties. Gold alloy exhibited the highest bacterial adhesion under similar roughness while this study showed no significant difference between titanium and zirconia. The results of titanium and zirconia resemble those in previous

study reporting that they are similar to each other in the pellicle composition and bacterial binding property. Other *in vivo* study also indicated that no differences in early bacterial colonization were found between the two materials (10). On the contrary, zirconia was reported to accumulate significantly fewer bacteria than titanium in another previous study (15). Such a disagreement may be attributed to the different experimental conditions, especially surface roughness, which is around 0.75 μm in R_a (15). Further studies are needed to verify the effect of material on the bacterial adhesion depending on the surface roughness.

The various factors affecting bacterial adhesion on solid surfaces include bacterial, substratum, and suspending medium characteristics. Among the substratum factors, SFE and surface roughness are two key factors in the initial adhesion and retention of oral bacteria. Roughening the surface affects the contact angles, thus also their SFE (16). Related to surface roughness, a previous study demonstrated that smoothing the surface under the threshold R_a value (≒0.2 ㎛) showed no further significant reduction in bacterial adhesion (17). If the R_a of the solid surface is less than 0.1 ㎛, the contact angle is not contingent on the surface roughness (18). This study adopted the idea that controlling the threshold R_a window excluded the distorting effect of surface roughness on

bacterial adhesion and contact angle and, in doing so, focused on the physicochemical properties of the material itself as a factor influencing bacterial adhesion.

In the oral environment, the process of biofilm formation on solid surfaces involves several progressive stages. The initial stage is the formation of a conditional film, coalesced entity of salivary proteins and cell-free enzymes (19). Further adhesion of successive microbes follows biofilm maturation. Therefore, to mimic the intraoral environment, the specimen surfaces of the present study were inoculated with sterile saliva for 2 hr to obtain a coating on the salivary pellicle. Previous studies demonstrate that pellicle coating does not completely nullify the inherent chemical characteristics of the surface even though it exhibits some homogenizing effects on the SFE (20). This was also the case in this study, and the aspects of initial bacterial adhesion varied according to the substrate even under controlled surface roughness and with the same saliva coating.

The initial bacterial adhesion is governed by physicochemical interactions (21). In the results of this study, gold specimens, which showed the highest polar surface energy and the lowest nonpolar surface energy, displayed the strongest bacterial adhesion. These findings contrast to the results of Sardin *et al.* which reported that bacterial adherence is re-

lated to the nonpolar component of the SFE of the substrate (22). Such contrast may be due to the large difference in total surface energy.

Quantification of biofilm formation can be carried out with various techniques such as the determination of the colony-forming unit, high resolution microscopic techniques, and staining techniques. From these techniques, staining assays using CV and the subsequent measurement of the absorbance is primarily used for monitoring biofilm *in vitro* (23). In a recent study, a resazurin assay and a similar assay using fluorescein diacetate were the best alternatives for microbial biofilm quantification (24). The two experimental methods quantifying attached bacteria derived a similar tendency in the result. Both of the techniques, staining and fluorometric, can be considered reproducible in the quantification of biofilm formation on several restorative materials. The results obtained through the staining technique showed a statistically significant difference between zirconia and titanium in experiments with *S. sanguinis* and *S. gordonii*. On the contrary, such differences were not detectable in the results obtained by the resazurin assay. This leaves the need for further investigation on whether the different result sets were driven by the inconsistency between total bacterial amount and metabolically active cell amount.

Despite the study's effort to mimic the conditions of the oral environment, bacterial adherence *in vitro* may differ in some ways from *in vivo* adherence. Even when surface roughness was set so low that it remained similar in each case, the amount of attached bacteria varied according to the materials in the *in vitro* study. To elucidate the relationship between oral bacteria and restorative materials, further *in vitro* studies are needed with a larger number of bacterial species, in addition to an expansive *in vivo* study.

In conclusion, different materials with extremely low surface roughness exhibit different amounts of bacterial adhesion. Zirconia and titanium may be more suitable than gold alloy as an abutment material, considering that they decrease the initial bacterial attachment on their surfaces, which clinically means that they lower the possibility of periimplantitis.

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Figures

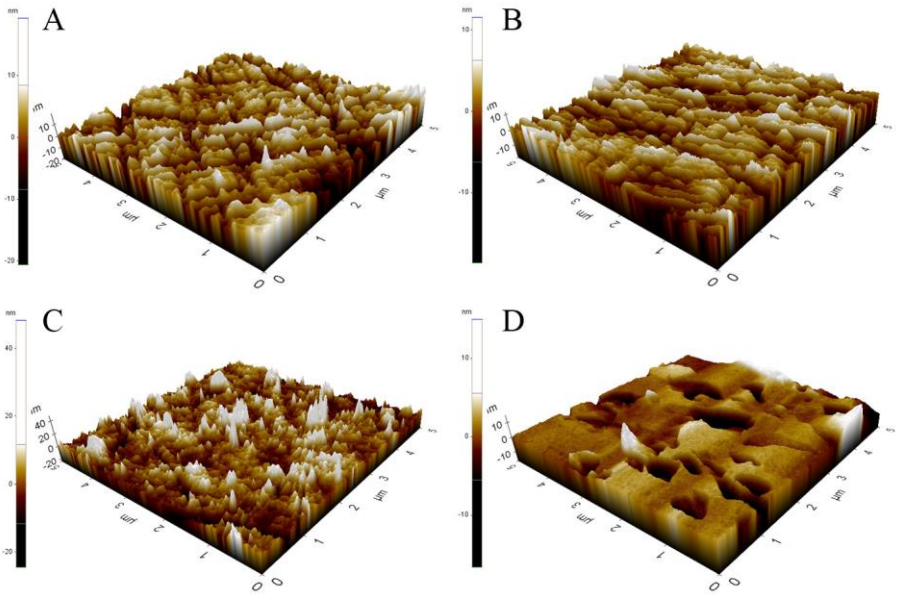

Fig. 1. Atomic force microscopic (AFM) images with average roughness values (R_a) of the four different implant materials: Zr (A), Zr-Al (B), Au (C), and Ti (D). Surface topography in nano-scale showed high frequency fine irregularities.

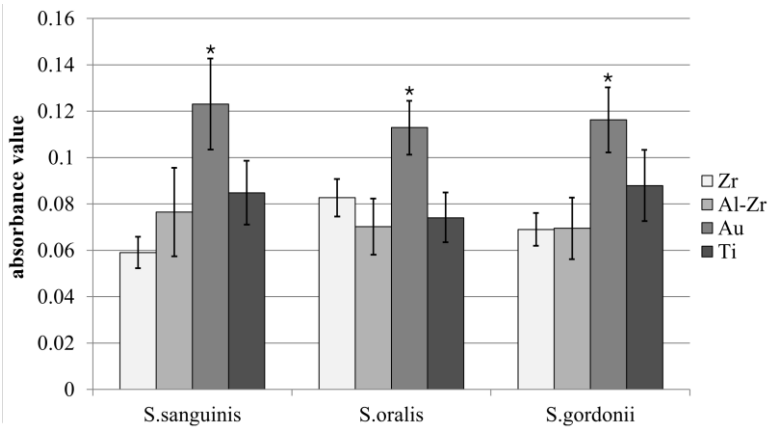

Fig. 2. Crystal violet absorbance for streptococcal adhesion. Zr, 3y-TZP; Al-Zr, alumina-toughened zirconia; Au, gold alloy; Ti, titanium. Asterisk indicates values that are significantly different (p <.001)

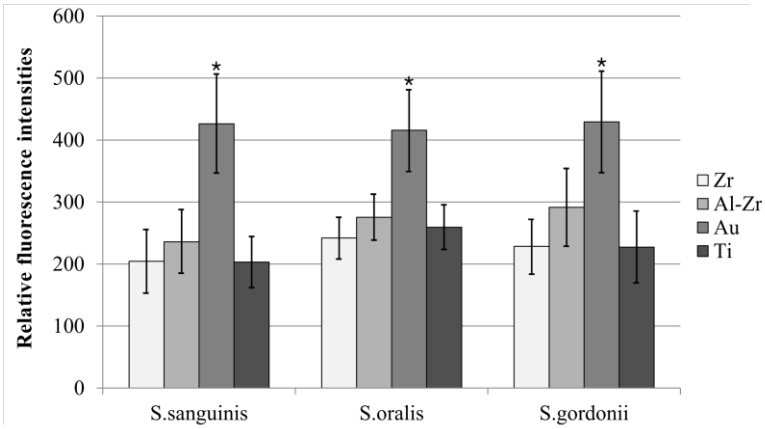

Fig. 3. Relative fluorescence intensities. Abbreviations are as in Fig. 2. Asterisk indicates values that are significantly different (p <.001)

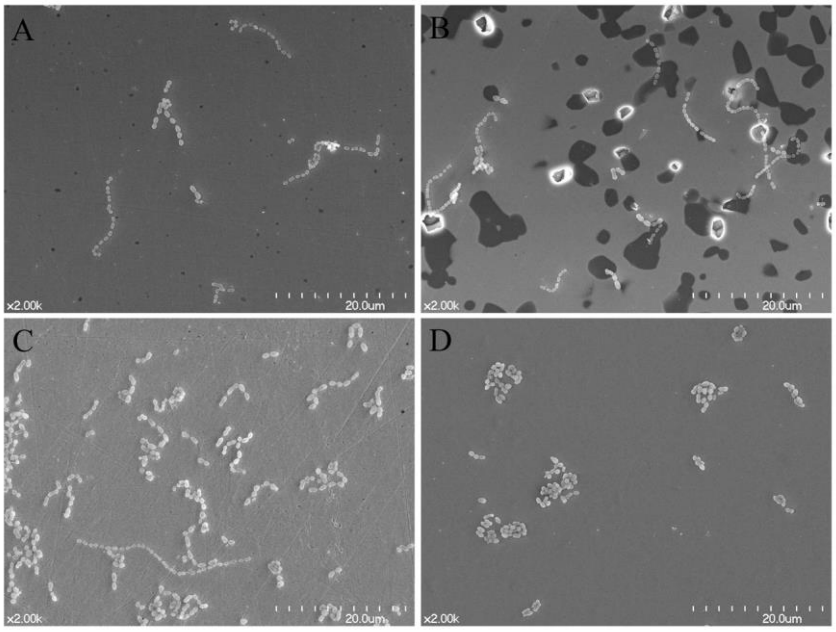

Fig. 4. Scanning electron microscopy of bacterial adhesion on the surface of Zr (A), Zr-Al (B), Au (C), and Ti (D). Adhered *S. oralis* exhibited a streptococcal chain. Scale bar represents 20 ㎛.

Tables

Table 1. Three early-colonizing streptococcal strains

Species	**Strain**
Streptococcus oralis	ATCC 9811
Streptococcus gordonii	ATCC 10558
Streptococcus sanguinis	NCTC 10904

Table 2. Mean values of parameters for surface roughness.

Material	R_a(nm)	R_q(nm)	R_z(nm)	R_{pv}(nm)
Zr	3.355	4.317	38.056	39.845
Al-Zr	2.460	3.190	28.638	30.206
Au	2.885	3.859	44.635	48.418
Ti	1.844	2.810	30.339	31.610

Table 3. Mean values and standard deviations of the contact angle measurements, Lifshitz van der Waals (γs^{LW}), polar (γs^{AB}) surface energy components, and total surface energies (γs) of each substrate (mJ/m^2), calculated according to the Owens-Wendt method.

Material	Zirconia	Al-Zr	Gold	Titanium
Contact angle				
Water	73.90(0.25)	73.32(0.71)	57.14(0.64)	65.53(1.15)
Formaldehyde	56.39(0.97)	44.94(2.02)	37.76(0.66)	45.81(1.56)
Energy (mJ/m^2)				
γs	37.88(0.87)	33.89(0.54)	59.92(1.08)	47.67(1.46)
γs^{LW}	0.35(0.11)	1.92(0.22)	0.04(0.01)	0.24(0.05)
γs^{AB}	37.53(0.98)	31.97(0.57)	59.88(1.09)	47.43(1.49)