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Bacterial Colonization of Zirconia Ceramic Surfaces: An In Vitro and In Vivo Study

Lia Rimondini, DDS¹/Loredana Cerroni, PhD, DDS²/Antonio Carrassi, DDS, MD³/Paola Torricelli, PhD⁴

Purpose: The microbial colonization of new ceramic materials developed for abutment manufacturing was assessed. **Materials and Methods:** The materials used in these experiments were disks of "as-fired" and "rectified" ceramic material made of tetragonal zirconia polycrystals stabilized with yttrium (Y-TZP) and commercially pure grade 2 titanium (Ti) with corresponding eluates. They were tested in vitro with the following bacteria: Streptococcus mutans, S sanguis, Actinomyces viscosus, A naeslundii, and Porphyromonas gingivalis. Proliferation was evaluated on plates by inhibitory halos around pits, previously inoculated with eluates obtained from the materials. Bacterial adhesion on materials was quantified by spectrophotometric evaluation of the slime production by the same bacteria. Moreover, early bacterial adhesion was evaluated in human volunteers and observed with SEM. **Results:** No inhibition of bacterial proliferation using eluates was observed. In vitro as-fired and rectified Y-TZP showed significantly more adherent S mutans than did Ti disks, while S sanguis seemed to adhere easily to Ti specimens. No differences were noted for Actinomyces spp and P gingivalis. In vivo Y-TZP accumulated fewer bacteria than Ti in terms of the total number of bacteria and presence of potential putative pathogens such as rods. No differences were observed between rectified and as-fired Y-TZP. **Discussion:** Overall, Y-TZP accumulates fewer bacteria than Ti. **Conclusion:** Y-TZP may be considered as a promising material for abutment manufacturing. (INT J ORAL MAXILLOFAC IMPLANTS 2002;17:793-798)

Key words: bacteria, bacterial colonization, dental abutment, dental implant, plaque, zirconia

Suprabony tissues, prosthetic structures, and the oral environment play pivotal roles in achieving and maintaining dental implant osseointegration. Transgingival implant components seem to be particularly important because their characteristics are essential in the prevention of failures and, at the same time, can be technologically controlled. Actually, joint strength and stability, anti-rotational

effect, and sealing at the implant-abutment connection are achieved because of the rheologic and manufacturing properties of implant abutments and connecting screws,^{1,2} while the external morphologic characteristics of implant-abutment surfaces may promote or delay plaque accumulation^{3,4} and consequently may help promote or prevent peri-implant disease.⁵⁻⁷ In addition, the material composition of transgingival components seems to influence the formation of epithelial attachment.^{8,9} Their shape and profile are able to guide gingival contouring^{10,11} and, together with the color of the material, strongly influence the final esthetic results of dental implant restorations.

Ceramic transgingival components have been introduced by many manufacturers to provide clinicians with more esthetic abutments than those fabricated from titanium (Ti). However, since these products are generally made of very stiff material such as alumina, they are often affected by unpleasant technologic problems because of their low resistance to bending forces.¹²

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Table 1 Profilometric Mean Values

Material	Mean Ra (μm)	Mean Rtm (μm)
Machined grade 2 Ti	0.22	2.16
As-fired Y-TZP	0.18	2.16
Rectified Y-TZP	0.04	1.14

Much attention has been recently focused on other ceramic materials commonly used in orthopedics, such as zirconia ceramic, which combines biocompatibility, pleasant esthetics, and impressive resistance to fractures.¹³ However, no information is available on how the oral plaque colonizes zirconia surfaces, although such interaction may influence the success or clinical failure of tissue integration. In fact, plaque accumulation has been suggested to be one of the major causes of implant failure. Anaerobic and Gram-negative species have been observed to often be associated with peri-implant disease.¹⁴

The aim of the present in vitro and in vivo study was to monitor and compare oral bacterial colonization on the surfaces of disks fabricated from machined grade 2 Ti and tetragonal zirconia polycrystal stabilized with yttrium (Y-TZP), which were obtained using various technologies.

MATERIALS AND METHODS

Sample Preparation

Sintered samples of Y-TZP were prepared from 3YB Tosoh powder (Tosoh, Tokyo, Japan) in a liquid suspension, initially pressed uniaxially at 77 MPa to obtain a cylinder fired in air at 1,500°C. The cylinder, 6 mm in diameter, was cut into 1-mm-thick disks using a diamond rotating wheel. The "as-fired" disks were ground and polished using a diamond rotating wheel.

An aliquot was flattened and highly polished ("rectified") on a flat surface using diamond rotating wheels with different grits in N₂ liquid atmosphere. Disks of similar dimensions were obtained by cutting a bar of grade 2 commercially pure Ti. The Ti disks were cut and polished using diamond wheels and grinding papers. Specimens were morphologically characterized with a laser profilometer (RM 600, Rodenstock, Gottingen, Germany) with the cutoff set at 0.25 mm and evaluation length at 2.0 mm. The arithmetic mean of the departures of the profile from the mean line (Ra) and the average of the 5 largest peak-to-valley heights in the evaluation length (Rtm) were recorded. Profilometric data are reported in Table 1.

All specimens were sterilized using ethylene oxide.

An aliquot each of both Ti and as-fired and rectified Y-TZP specimens was incubated in phosphate-buffered saline (PBS) (0.2 g/mL) for 1, 3, and 5 days at 37°C to obtain extracting solutions according to ISO 10993-12:1998.

In Vitro Tests

Microorganisms and Growth Conditions. The organisms used were *Porphyromonas gingivalis* (ATCC25175), *Streptococcus mutans* (ATCC25175), *Streptococcus sanguis* (ATCC10556), *Actinomyces viscosus* (ATCC15987), and *Actinomyces naeslundii* (ATCC12104). All bacterial cultures were incubated anaerobically at 37°C.

Agar Bacterial Inhibition Assay. The agar bacterial inhibition assay is a modification of the method described by Tseng and Wolff.¹⁵ The strains were pre-cultured in Schaedler broth (1% wt/v Tryptone soy broth, Sigma-Aldrich Gallarate, Milan, Italy) for 18 hours, and diluted broth cultures, characterized by a spectrophotometric absorbance value of 0.2 recorded at 540 nm, were obtained. Then, a 1:50 dilution of the adjusted bacterial broth culture was obtained using Schaedler broth. Two hundred microliters of each broth dilution were uniformly flooded on agar plate containing Schaedler broth, 5% defibrinated sheep blood, 0.0005% hemin (Sigma-Aldrich Gallarate), and 0.00005% menadione (Sigma-Aldrich Gallarate). Four wells, each 0.65 mm in diameter, were made in each agar plate, and the pits were filled with 70 μL of the extracting solution from all specimens obtained at the different times. PBS was used as the negative control and a standard solution of 0.2% chlorhexidine (Sigma-Aldrich Gallarate) as the positive control. Afterward, cultures were incubated anaerobically for 4 days at 37°C. The diameter of the bacterial zone of inhibition was then measured using a gauge. The test was repeated 3 times (Fig 1).

Microbial Adhesion In Vitro Test. The amount of adherent bacteria was quantified through evaluation of the slime using N-lauryl-sarcosinate (Sarcosyl, Sigma-Aldrich Gallarate) as the extraction agent¹⁶ according to the following procedure. Standard inocula of 1 mL were obtained from broth cultures of the different strains after 18 hours. They were characterized by a spectrophotometric absorbance value of 0.4 recorded at 540 nm. Each inoculum was put into the pits of the 24-well plates, each containing the as-fired and rectified Y-TZP and Ti specimens. Plates were then incubated in an anaerobic environment at 37°C for 36 hours. After this period, specimens were removed, rinsed in PBS, and fixed in Bouin's solution

at 25°C for 2 hours. They were then rinsed again in PBS, incubated in crystal-violet, dissolved in 0.01% PBS for 15 minutes, and rinsed twice with PBS; finally, 200 µL of N-lauryl-sarcosinate 10% were added to each well.^{16,17} After 5 minutes of incubation at 37°C, the lysates were finally analyzed with a microspectrophotometer at 540 nm.

Seven specimens of each material were evaluated for each bacterial strain.^{16,17}

In Vivo Experiment

The experiment was performed after recruiting 10 volunteers ranging in age between 20 and 23 years with good oral and general health conditions and high standards of oral hygiene. Informed consent was obtained from each participant. None had taken antibiotics or used an antibacterial solution during the 3 months prior to the experimentation.

Silicone stents were produced, and 3 specimens, 1 of each type, were mechanically fixed into the silicone during its polymerization. A stent carrying the specimens was fixed onto the buccal region of the molar and premolar of each volunteer using orthodontic wires. After stent placement, the subjects suspended any oral hygiene procedures for 24 hours. The specimens were then removed and processed for scanning electron microscopy (SEM) (840A, Jeol, Tokyo, Japan) as follows: fixed in 2.5% glutaraldehyde water solution for 2 hours; buffered in sodium cacodylate 1 mol/L; rinsed in water; dehydrated in alcohol (50%, 70%, 80%, 90%, 100% for 10 minutes each); dehydrated at CO₂ top critical point in a bomb (Top Critical Point 30, W. Pabish, Pero, Milan, Italy); and finally coated with a 20-nm-thick gold-palladium layer in a coating unit (Coating Unit E5100, Polaron Equipment, Watford, United Kingdom). The specimens were evaluated with SEM as previously described using the secondary electron mode working between 5 and 15 Kv. A global area of 100 × 125 µm was examined for each specimen. The area was the sum of 5 fields of equal dimensions, which were randomly selected on the specimen surface using a grid. For each field the following variables were recorded: presence (= 1) or absence (= 0) of cocci, short rods (< 10 µm), and long rods (> 10 µm). The presence of occasionally adherent epithelial cells was similarly recorded. The cumulative values for each area were used for statistical evaluation. An index of the amount of bacteria was calculated for each area by summing the following scores recorded in each field: number of bacteria ≤ 5, score = 0; number of bacteria 6 to 30, score = 1; number of bacteria 31 to 100, score = 2; number of bacteria more than 100, score = 3.^{3,18}



Fig 1 Example of 1 assay using test materials (Titanium 4-T2P), positive control (0.2% chlorhexidine), and negative control (PBS).

Statistical Analysis

One-way analyses of variance (ANOVA) and Scheffé tests were used to compare slime data. Statistical analysis of in vivo experimentation was performed using data from the cumulative area with the Kruskal-Wallis ANOVA exact test followed by the Mann-Whitney *U* test to compare substrates with each other. The Monte Carlo method was used for probability calculation. The significance level was set at $P < .05$.

RESULTS

In Vitro Tests

Analysis of the bacteria assay showed no inhibition of bacteria proliferation. The behavior of all the eluates obtained from the different materials was comparable to that recorded with negative controls. However, differences were observed in the adhesion test with respect to the slime production (Table 2). *S mutans* was the most adherent strain, producing more slime on as-fired Y-TZP than on rectified and Ti specimens ($P < .01$). In contrast, *S sanguis* seemed to adhere to grade 2 Ti easily, with the *P* value approaching significance ($P < .1$). No differences were observed with regard to *Actinomyces* spp and *P gingivalis*.

In Vivo Experiment

Table 3 shows the index of bacteria density recorded on the specimen surfaces and the presence of the different morphotypes. Both Y-TZP surfaces accumulated significantly fewer bacteria than Ti, with a

Bacteria	As-fired Y-TZP (nm)	Rectified Y-TZP (nm)	Machined Ti (nm)	P (1-way ANOVA)
<i>S mutans</i>	0.48 \pm 0.02*	0.27 \pm 0.01*	0.33 \pm 0.01*	.01
<i>S sanguis</i>	0.09 \pm 0.01	0.13 \pm 0.01	0.18 \pm 0.01	NS
<i>A viscosus</i>	0.15 \pm 0.01	0.14 \pm 0.01	0.16 \pm 0.01	NS
<i>A naeslundii</i>	0.21 \pm 0.01	0.30 \pm 0.01	0.20 \pm 0.01	NS
<i>P gingivalis</i>	0.08 \pm 0.02	0.09 \pm 0.00	0.11 \pm 0.01	NS

NS = not significant.

* $P < .01$ (Scheffe test).

Material	BDI	Cocci	Short rods	Long rods	Keratinocytes
As-fired Y-TZP	6.0 \pm 4.9*	3.7 \pm 0.8	0.7 \pm 1.3*	0.1 \pm 0.4*	0.1 \pm 0.0
Rectified Y-TZP	8.3 \pm 3.1*	5.0 \pm 0.3	1.3 \pm 1.8	0.0 \pm 1.2*	1.0 \pm 0.6
Machined Ti	11.3 \pm 4.3*	4.3 \pm 1.2	3.3 \pm 1.8*	0.8 \pm 0.9*	0.1 \pm 0.0
P^{\dagger}	< .5	< .5	< .5	< .5	NS

NS = not significant.

* $P < .05$, comparison between zirconia and titanium substrates (Mann-Whitney U test with Monte Carlo method to compute probability).

† Kruskal-Wallis ANOVA exact test with Monte Carlo method to compute probability.

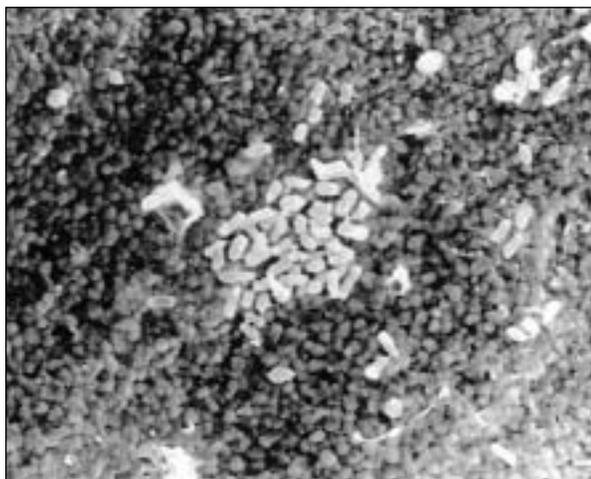


Fig 2 SEM micrograph of bacteria colonizing an as-fired Y-TZP surface. A cluster of few cocci is observable (original magnification $\times 6,000$; bar = 1 μm).

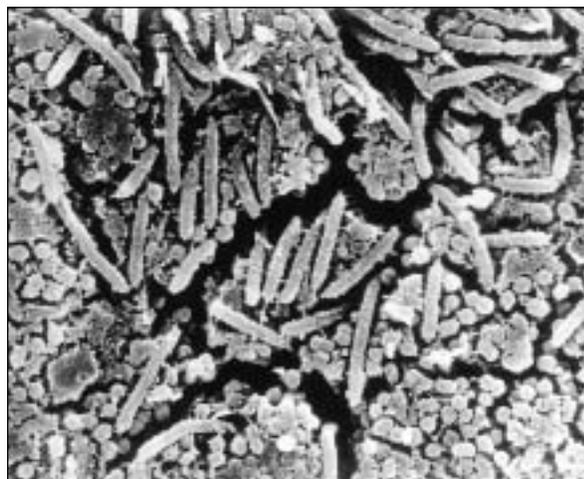


Fig 3 SEM micrograph of bacteria colonizing a Ti surface. An homogeneous layer of cocci covers the specimen surface. Rods are apparent (original magnification $\times 6,000$; bar = 1 μm).

prevalence of cocci and the absence of long rods. The Ti surfaces appeared more uniformly coated with a structured biofilm made of pellicle and bacteria (Fig 2), whereas the Y-TZP surfaces appeared colonized by clusters of bacteria (Fig 3). No differences were observed between as-fired and rectified surfaces in terms of the amount of bacteria and presence of morphotypes. Epithelial cells were observed on some rectified Y-TZP specimens.

DISCUSSION

The purpose of the present study was to evaluate in vitro the capability of different strains of oral bacteria to adhere to and grow on Ti and as-fired Y-TZP specimens whose Ra roughness values, ranging from 0.18 to 0.22 μm on average, may be comparable to abutments available commercially (observed to range from 0.10 to 0.30 μm).¹⁹ Adhesion and

growth on highly polished rectified Y-TZP surfaces, which are 2 times more flat and polished than those of commercially available abutments, were also evaluated. Additionally, this study included an in vivo experiment to investigate early colonization in the presence of composite plaque, salivary pellicle, and removal force related to salivary flow, muscles, and chewing activity.

No antimicrobial activity was detected for either Ti or zirconia materials using the killing curve test in vitro on the strains used. Such a finding is not surprising. The antimicrobial activity of Ti is very controversial and directly related to the concentration of Ti ions and test sensitivity.²⁰ No previous data are available on zirconia as far as oral strains are concerned.

The adhesion tests for the indirect evaluation of the biofilm amount by assessment of slime and lipopolysaccharide membranes detached by sarcosyl showed differences between Ti and ceramic surfaces. *S mutans* adhered to ceramic surfaces more easily than other pioneer bacteria, such as *S sanguis*, which seemed to have more affinity to Ti than to Y-TZP surfaces. Moreover, no differences were noted between *S sanguis* adhesion to highly polished rectified and as-fired Y-TZP surfaces. This latter observation is apparently at odds with common in vivo findings, according to which rougher surfaces accumulate more bacteria.²¹ This phenomenon is in fact generally enhanced in vivo because of the sheltering effect of the rough surfaces against the removal forces that are lacking under in vitro experimental conditions. Moreover, other factors, such as chemical and physical properties of the surfaces (surface wettability of materials combined with bacterial strains used), may overrule those chemico-physical aspects such as roughness,²² which may modify the surface chemical composition and wettability,^{23,24} especially in short-term tests such as the present experiment.

The use of an in vivo early colonization model offers the opportunity to evaluate materials in a simulated clinical condition presenting composite plaque, salivary pellicle, and removal forces. According to the present results, the Y-TZP surfaces accumulated fewer bacteria than Ti, with a prevalence of cocci, few short rods, and no long rods, which is suggestive of an immature plaque.²⁵

No significant differences were noted between as-fired and rectified zirconia in plaque colonization, although the rectified surfaces were smoother than the as-fired surfaces. A possible explanation may be the roughness threshold of clinical relevance (about Ra = 0.2 µm) observed in both early³ and long-term in vivo models.⁴

Finally, the many ectopic epithelial cells occasionally observed on Y-TZP surfaces seem to suggest that Y-TZP might be a promising material capable of enhancing epithelial attachment formation, although this hypothesis has not been tested.

CONCLUSION

Within the limits of this study, zirconia ceramic may be a suitable material for manufacturing implant abutments, with a low bacterial colonization potential. High polishing of zirconia ceramic does not seem to offer any advantages over the as-fired material in terms of colonization.

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