

RESEARCH AND EDUCATION

Effect of the aging of titanium and zirconia abutment surfaces on the viability, adhesion, and proliferation of cells and the adhesion of microorganisms



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Material surface changes may be involved in the direct contact of peri-implant tissue with a dental implant abutment. The abutment material undergoes changes with the aging and degradation processes.¹⁻³ Titanium (Ti) is the most commonly used material for implant abutments with its excellent biocompatibility and corrosion resistance^{4,5} and the high chemical stability of the surface oxide layer.⁶ However, the biological aging of a titanium surface has been associated with a markedly reduced adhesion of proteins and cells from the progressive accumulation of hydrocarbons on the titanium surface.⁵

ABSTRACT

Statement of problem. The longevity of dental implants depends on the maintenance of peri-implant tissue and absence of inflammation. How the physical-chemical properties intrinsic to each material over time can affect adhesion, given constant cell turnover and biofilm development, remains unclear.

Purpose. The purpose of this in vitro study was to evaluate the influence of aging on the viability, adhesion, and proliferation of normal oral keratinocytes (Nok-si) and on the multispecies biofilm formation of *Fusobacterium nucleatum* (*F. nucleatum*), *Porphyromonas gingivalis* (*P. gingivalis*), and *Streptococcus sanguinis* (*S. sanguinis*).

Material and methods. Zirconia (ZrO₂) and titanium (Ti) disks were analyzed by surface roughness, water contact angle, and X-ray diffraction before and after aging in an autoclave. The Nok-si cell viability was evaluated by using a 3-(4,5-dimethylthiazole-2-yl)2,5-diphenyl tetrazolium bromide assay (MTT), morphology by scanning electron microscopy (SEM), and proliferation and adhesion by using a confocal microscope. Multispecies biofilms were analyzed quantitatively by colony-forming units per milliliter (CFU/mL) and qualitatively by SEM.

Results. For Ti, the aging process affected the roughness and wettability. However, for ZrO₂, the aging did not affect roughness but did affect wettability and the ratio of the tetragonal to monoclinic phase ($P < .05$). A significant difference was found in the bacterial growth for Ti (nonaged and aged) in relation to the control, and no differences were found in Ti before and after aging; however, ZrO₂ had increased growth of microorganisms after aging. For ZrO₂, a statistically significant difference was found between aged ZrO₂ and the control ($P < .001$).

Conclusions. The results indicate that, after the aging, Ti showed better cell adhesion and proliferation and lower biofilm adhesion than zirconia. (*J Prosthet Dent* 2019;122:564.e1-e10)

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Clinical Implications

The findings could offer a starting point to selecting the appropriate material for abutments and for the gingival profile of each patient by considering the costs, the longevity of the material, and tissue recovery. Titanium showed better cell adhesion and proliferation and lower biofilm adhesion than zirconia.

Zirconia (ZrO_2) appears to be a promising alternative abutment material because of its high mechanical and flexural strength and excellent esthetic properties.⁷ The in vivo degradation of ZrO_2 has been reported to have high variability as a consequence of the strong influence of this aging process.¹⁻³ Low-temperature degradation (LTD) or aging results in the transformation of a metastable tetragonal structure (t) into a stable monoclinic structure (m) in yttria-stabilized tetragonal zirconia (Y-TZP). LTD can be simulated in an autoclave at 134 °C under 200 kPa for a period of 20 hours.^{8,9} This accelerated aging protocol has been recommended because Chevalier et al¹⁰ observed that 20 hours is sufficient to promote an extensive t→m transformation (approximately 80%). Thus, this protocol could evaluate cellular behavior on the surfaces of aged Ti and ZrO_2 .

Chemical changes resulting from the natural aging process alter the physical-chemical material properties, including roughness, wettability, surface free energy (SFE), and the composition and phase of the material.^{4,11-20} Reduced epithelial downgrowth and more coronally located connective tissue adaptation has been reported for rough surfaces²¹; thus, roughness below 0.2 mm might be the most effective way to obtain a protective barrier around the abutments.^{16,19,22,23} If crystallographic phase transformation in the zirconia occurs, the surface becomes altered and consequently may interfere with cellular adhesion.^{1,12,20}

SFE can also play an important role in the adsorption of proteins and cell adhesion and distribution.^{4,24} Thus, the purpose of this study was to evaluate the effect of in vitro aging on the physicochemical properties of Ti and ZrO_2 abutment materials. The effects of aging on the viability, adhesion, and proliferation of normal oral keratinocytes (Nok-si) and on the multispecies biofilm formation of *Fusobacterium nucleatum* (*F. nucleatum*), *Porphyromonas gingivalis* (*P. gingivalis*), and *Streptococcus sanguinis* (*S. sanguinis*) were also evaluated. The null hypothesis tested was that aging of the abutment materials, Ti and ZrO_2 , did not influence the viability, adhesion, and proliferation of Nok-si or the multispecies biofilm formation of *F. nucleatum*, *P. gingivalis*, and *S. sanguinis*.

MATERIAL AND METHODS

Figure 1 represents the flowchart of the study. Disk-shaped specimens of Ti and ZrO_2 (Conexão Sistemas de Próteses Ltda)^{17-19,25} with a diameter of 8 mm and thickness of 2 mm were obtained (N=45). Surface roughness and SFE were analyzed according to protocols described previously.^{17,19} The disks used had an average roughness (Ra) between 0.1 and 0.2 μm .^{22,23} For SFE, the sessile drop technique was used to measure the contact angles of liquid drops (distilled water, ethylene glycol, polyethylene glycol, and diiodomethane). SFE was calculated in accordance with the Owens-Wendt-Rabel-Kaelble (OWRK) method.^{18,19,26} X-ray diffraction (XRD) was performed by using an XRD device (D 5000; Siemens Corp) with a monochrome beam of Cu Ka (15 418 Å) filtered with nickel.^{1,27} The disks were cleaned by immersion in acetone for 20 minutes, immersion in test tubes containing distilled water, ultrasonic cleaning for 20 minutes, and thorough wash with distilled water.²⁸

LTD was simulated in an autoclave (AB-25|AB-42; Phoenix Lufenco) at 134 °C for 20 hours and a pressure of 200 kPa (ISO 14801:2007) (n=29).^{9,10,29} After the aging process, the same disks were resubmitted to the roughness, SFE, and XRD analyses. The control disks were sterilized overnight by gamma irradiation at 25 kGy from an artificial cobalt-60 source in a nuclear reactor (ISO-11137: 2006) (Energy and Nuclear Research Institute, IPEN).¹⁷

Nok-si were cultured in DMEM with 4.5 g/L of glucose (Invitrogen; Thermo Fisher Scientific), supplemented with fetal bovine serum, penicillin (1000 IU/mL), streptomycin (10 mg/mL), and amphotericin (25 μg /mL).³⁰ Cells were cultured at 37 °C with 5% CO_2 in a humidified atmosphere and used between passages 3 and 7. For analysis, 3×10^4 Nok-si were plated on aged and nonaged Ti and ZrO_2 specimens, according to the protocol for each analysis. The 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich Co) assay was performed according to the protocol described previously.¹⁹

The spectrophotometric measurements were performed at 562 nm (EZ Read 400; Biochrom Ltd) by using isopropanol as a control. The experiments were performed at 3 different times in triplicate (n=9). The morphology of the cells adhered to the specimens (aged and nonaged surfaces) was evaluated after 24 hours in a 24-well plate. The specimens were fixed with 400 μL of 2.5% glutaraldehyde (Sigma-Aldrich Co) overnight, washed 3 times with sterile PBS, subjected to gradual dehydration in alcohol solutions (70%, 90%, and 100%), transferred to stubs, and stored in a desiccant for at least 7 days.¹⁹ For analysis, the specimens were coated with gold and analyzed by scanning electron microscopy

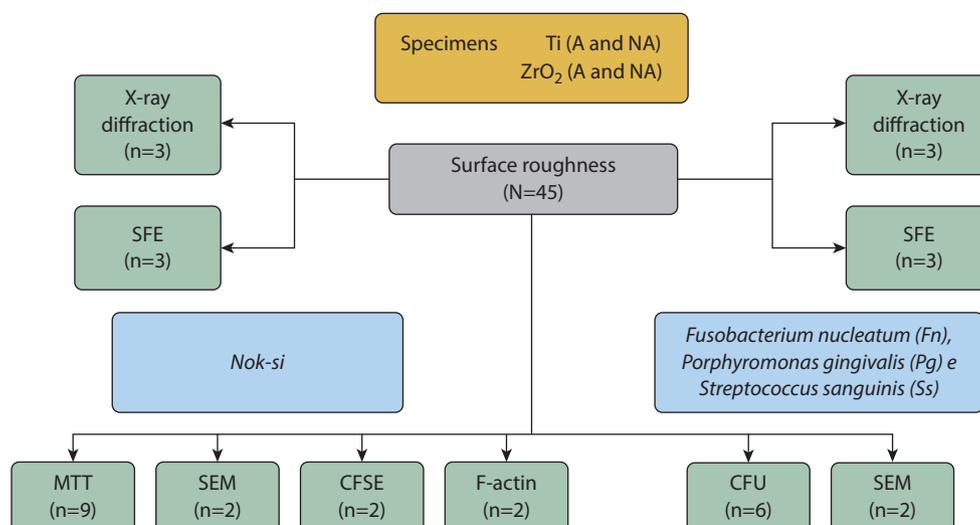


Figure 1. Flowchart of study. A, aged; CFSE, carboxyfluorescein succinimidyl ester; CFU, colony-forming units per milliliter; MTT, 3-(4,5-dimethylthiazole-2-yl)2,5-diphenyl tetrazolium bromide assay; NA, nonaged; SEM, scanning electron microscopy; Ti, titanium; ZrO₂, zirconia.

(SEM) (JSM-6610LV; JEOL Ltd). This assay was performed in duplicate (n=2).

To assess the cellular proliferation of Nok-si, on specimens, the cells were stained with carboxyfluorescein succinimidyl ester (CFSE) (5 μ M) (Thermo Fisher Scientific) and incubated at 37 °C for 20 minutes. Then, 60 μ L of PBS was added to stop the CFSE reaction, and 2 washes were carried out for 5 minutes. The cells were then seeded on each disk in a 48-well plate and incubated for 24 and 48 hours. The analysis was performed by using a confocal microscope (LSM 800; Carl Zeiss) (n=2): laser wavelength: 488 nm at 5.5%; detection wavelength: 488 to 520 nm; detection gain: 700 V. In another assay, the specimens were washed with 300 μ L of 1 \times PBS, and then the cells were fixed with 300 μ L of 4% paraformaldehyde for 20 minutes and washed again with PBS. Then, 0.1% Triton (Sigma-Aldrich Co) was added for 15 minutes. Two washes were carried out with PBS, and then 2 drops of Actin Red (ActinRed 555 ReadyProbes Reagent; Thermo Fisher Scientific) reagent were added to each specimen. Then, 300 μ L of 0.1% Hoechst (33342; Life Technologies; Thermo Fisher Scientific) was added and incubated for 10 minutes. The specimens were washed once with 300 μ L of PBS, and then each specimen remained in 300 μ L of PBS until analyzed. The cells were evaluated by using a confocal microscope (LSM 800; Carl Zeiss) (laser wavelength: 405 nm at 3.51%; detection wavelength: 400 to 555 nm; detection gain: 896 V; laser wavelength: 561 nm at 1.10%; detection wavelength: 555 to 700 nm; detection gain: 630 V).

Multispecies biofilms were generated by using the method described by Sánchez et al.³¹ *F nucleatum* (ATCC 25586), *P gingivalis* (ATCC 33277), and *S sanguinis* (ATCC 10556) were grown on Brucella supplemented with hemin

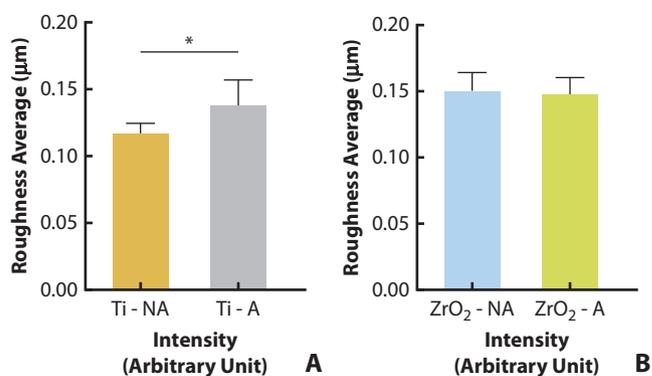


Figure 2. Effect of aging on surface roughness: A, Roughness average for Ti; B, Roughness average for ZrO₂. A, aged; NA, nonaged; Ti, titanium; ZrO₂, zirconia. Data shown as mean \pm standard deviation (n=10). $P < 0.05$ was considered statistically significant. * $P = .003$.

(5 mg/mL), menadione (1 mg/mL) (Merck KGaA), and 5% sheep deliberated blood for 3 days in an anaerobic chamber (atmosphere of 85% N₂, 10% to 15% H₂, and 5% to 10% CO₂) at 37 °C^{17,32,33} to confirm the purity of the strains. The logarithmic growth phase (Log) was determined for each bacterium; thus, colonies were transferred to 96-well plates, with a final volume of 200 μ L of supplemented Brucella broth. The concentrations used ranged from 10⁷ to 10⁹ UFC/mL, and the times measured were 0, 6, 12, 24, 36, 48, and 72 hours after incubation in an anaerobic chamber at 37 °C. After growth curve graphs were plotted with the Prisma program, the best concentration and incubation time for the Log phase of each bacterium were determined (data not shown). For the establishment of the multispecies biofilm, after 30 hours, 1 disk per well was placed in a 48-well plate and submerged in 100 μ L of Brucella broth containing each bacterium at a concentration of 10⁹ CFU/mL, for a total

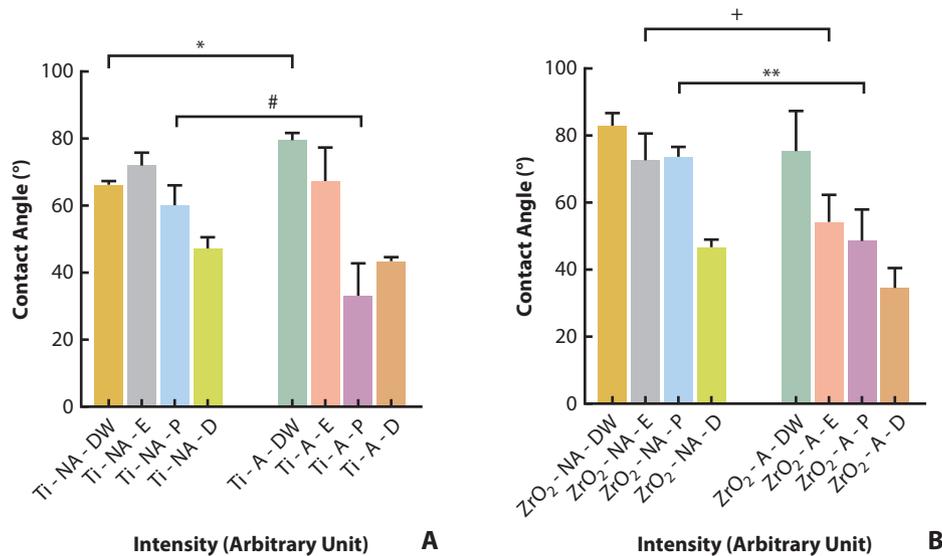


Figure 3. Effect of aging on wetting property. A, Contact angle for Ti; B, Contact angle for ZrO₂. A, aged; D, diiodomethane; DW, distilled water; E, ethylene glycol; NA, nonaged; P, polyethylene glycol; Ti, titanium; ZrO₂, zirconia. A two-tailed paired test performed by using Graph-Pad Prism version 5.0c. Data shown as mean ±SD (n=3), and P<.05 considered statistically significant. *P=.011; #P=.044; +P<.001; **P=.035.

Table 1. Titanium and zirconia surface free energy before and after aging

Surface Free Energy	Nonaged	Aged
Ti	35.57 mN/m	32.60 mN/m
ZrO ₂	25.36 mN/m	34.86 mN/m

Ti, titanium; ZrO₂, zirconia.

volume of 300 μL (*E. nucleatum*, *S. sanguinis*, and *P. gingivalis*).³¹ After the adhesion period (72 hours), the disks were washed twice with sterile PBS to remove any bacteria that were not adhered.³³⁻³⁶ Then, the disks were removed from the plate, and protocols of each analysis were performed. Three polystyrene disks for cell adhesion were used as controls.

For the colony-forming unit (CFU/mL), the disks were placed into Falcon tubes with 500 μL of PBS and kept in an ultrasound bath for 20 minutes (Ultrasonic Cleaner 1440 Plus; ODONTOBRAS) to detach the biofilm from the specimens. Then, 100 μL of the bacterial suspension was serially diluted in hemi-mandarin and blood for the subsequent determination of the bacterial growth and plaques maintained under anaerobiosis by using a digital colony counter. The bacterial growth was calculated as CFU/mL. Fifty-microliter aliquots were plated on Brucella agar, and the plates were incubated in an anaerobic chamber at 37 °C for 72 hours. After the incubation period, counting was performed by an investigator blinded to the experimental design.^{33,35,36} This assay was performed in triplicate. The morphological analysis of the adhered biofilm was performed by SEM (JEOL JSM-6610LV), and the dehydration and fixation protocols were followed as previously described.^{17,31}

The data were analyzed by using a software program (Graph Pad Prism; GraphPad Software Inc) (α=.05). The surface roughness data showed adherence to a D’Agostino normal curve, and a Pearson normality test was applied and complemented with a paired 2-tailed t test. For SFE, a 2-tailed paired test was performed. A paired t test was performed to determine if there was a difference in roughness and SFE measurements before and after the aging process. For the cellular viability data (MTT), a 1-way ANOVA was applied and complemented with a post hoc Tukey test. The CFU/mL data were analyzed by using a 1-way ANOVA with a post hoc Tukey test.

RESULTS

The roughness data are shown in Figure 2. The results of a 2-tailed paired test concluded that the aging process did not affect the average roughness of the ZrO₂ disks. A statistically significant difference was found between the roughness measurements of the Ti surface before and after the aging process (P=.003).

The aging process affected the wettability of the ZrO₂ surface when the surface was in contact with ethylene glycol (P<.001) and polyethylene glycol (P=.035). The aging process affected the wettability of the Ti surface when the surface was in contact with distilled water (P=.011) and polyethylene glycol (P=.044) (Fig. 3). The SFE value of ZrO₂ and Ti, aged and nonaged, is shown in Table 1. The results of the X-ray diffraction analysis of the aged and nonaged ZrO₂ and Ti disks are shown graphically in Figure 4. For ZrO₂, aging in the autoclave was shown to increase the amount of the monoclinic phase. No changes were observed for Ti.

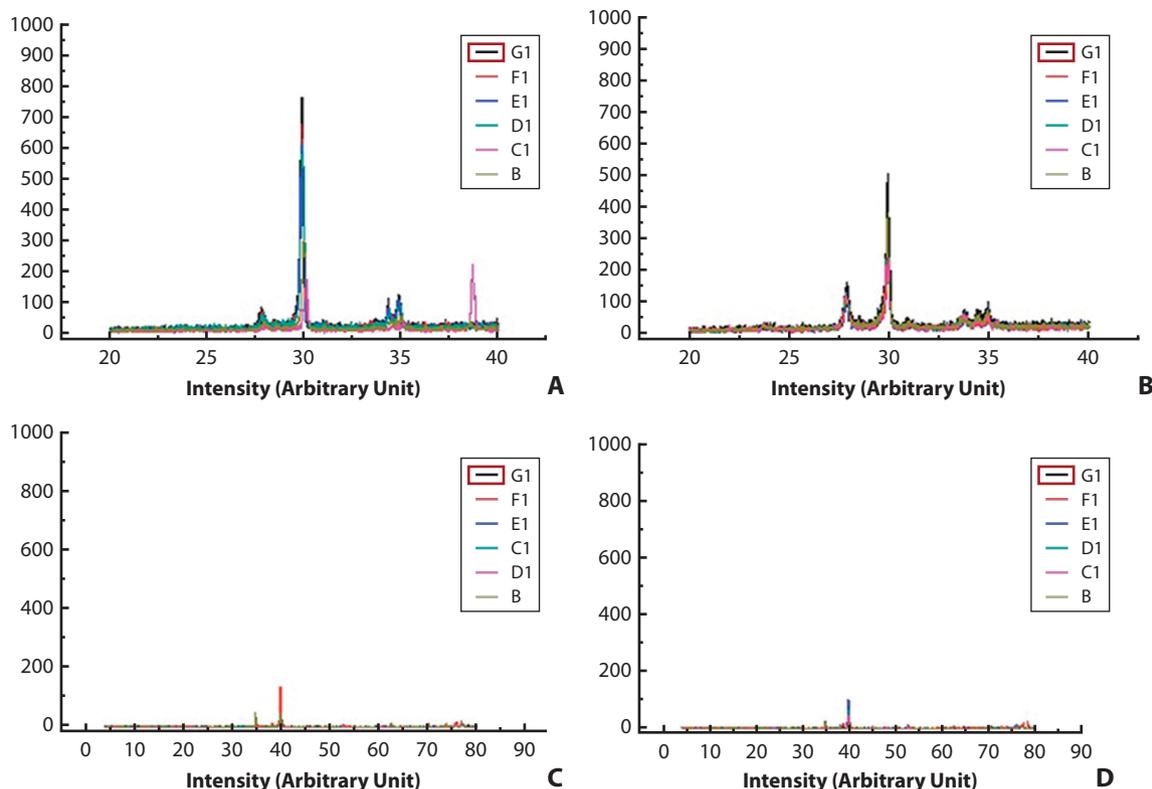


Figure 4. Diffraction (XRD) patterns of nonaged and aged surfaces of zirconia and titanium. A, ZrO₂ nonaged. B, ZrO₂ aged. C, Ti nonaged. D, Ti aged. A, aged; NA, nonaged; Ti, titanium; ZrO₂, zirconia; B, C1, D1, E1, F1, and G1 represent replicates of nonaged and aged Ti and ZrO₂ specimens.

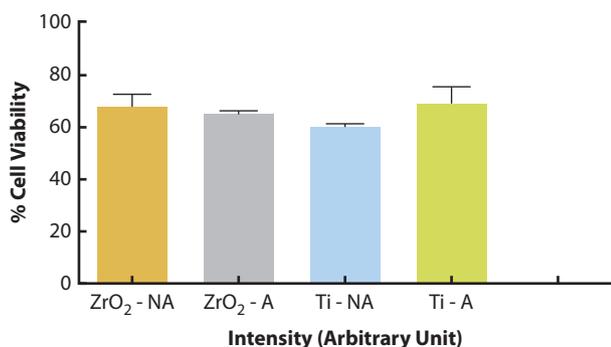


Figure 5. Viability of normal oral keratinocytes (% of control-GCS) after adhesion to nonaged (ZrO₂-NA/Ti-NA) or aged (ZrO₂-A/Ti-A) zirconia and titanium surfaces compared with control. Columns represent means, and error bars represent standard deviations. A, aged; NA, nonaged; Ti, titanium; ZrO₂, zirconia.

The cell viability data (MTT assay) are graphically presented in Figure 5. The results of the 1-way ANOVA concluded that there were no statistically significant differences in the viability of the different nonaged and aged materials ($P > .05$), which had no effect on cell viability or on the interaction between them (aging material). Therefore, the viability of Nok-si was not affected by the type of material on which they were cultured. Similarly, there was no change in Nok-si viability on aged and nonaged surfaces.

Figure 6 shows the morphology of Nok-si cultured on ZrO₂ and Ti before and after aging. The cell density after adhesion was qualitatively similar in the aged and nonaged ZrO₂ and Ti substrates. On the Ti surface (nonaged and aged), the cells had an increased number of projections and were scattered on the surfaces, while on the ZrO₂ surface, the cells had a rounded conformation and few projections. Figure 7 shows the proliferation of Nok-si after 24 and 48 hours of adhesion to the different ZrO₂ and Ti substrates, both nonaged and aged. The proliferation of cells on Ti was higher than that on ZrO₂, especially on the aged surfaces after 48 hours. The cells proliferated less on both the nonaged and aged ZrO₂ surfaces than on the Ti surfaces; however, in the ceramic setting, no significant increase in cell proliferation was observed between 24 and 48 hours.

Figure 8 shows the labeled β -actin of the cytoskeleton of the adhered Nok-si, revealing the interaction of the cells with the studied surfaces. For both materials and under the conditions evaluated, more β -actin were labeled after the 24-hour incubation than at the 48-hour timepoint; however, the relative fluorescence intensity of the cells on the Ti surfaces (aged and nonaged) was significantly higher than that on the tested ZrO₂ surfaces. A similar behavior was maintained at 48 hours; however, a difference was observed. At 48 hours, the reduction in the fluorescence intensity of the ZrO₂ specimens was

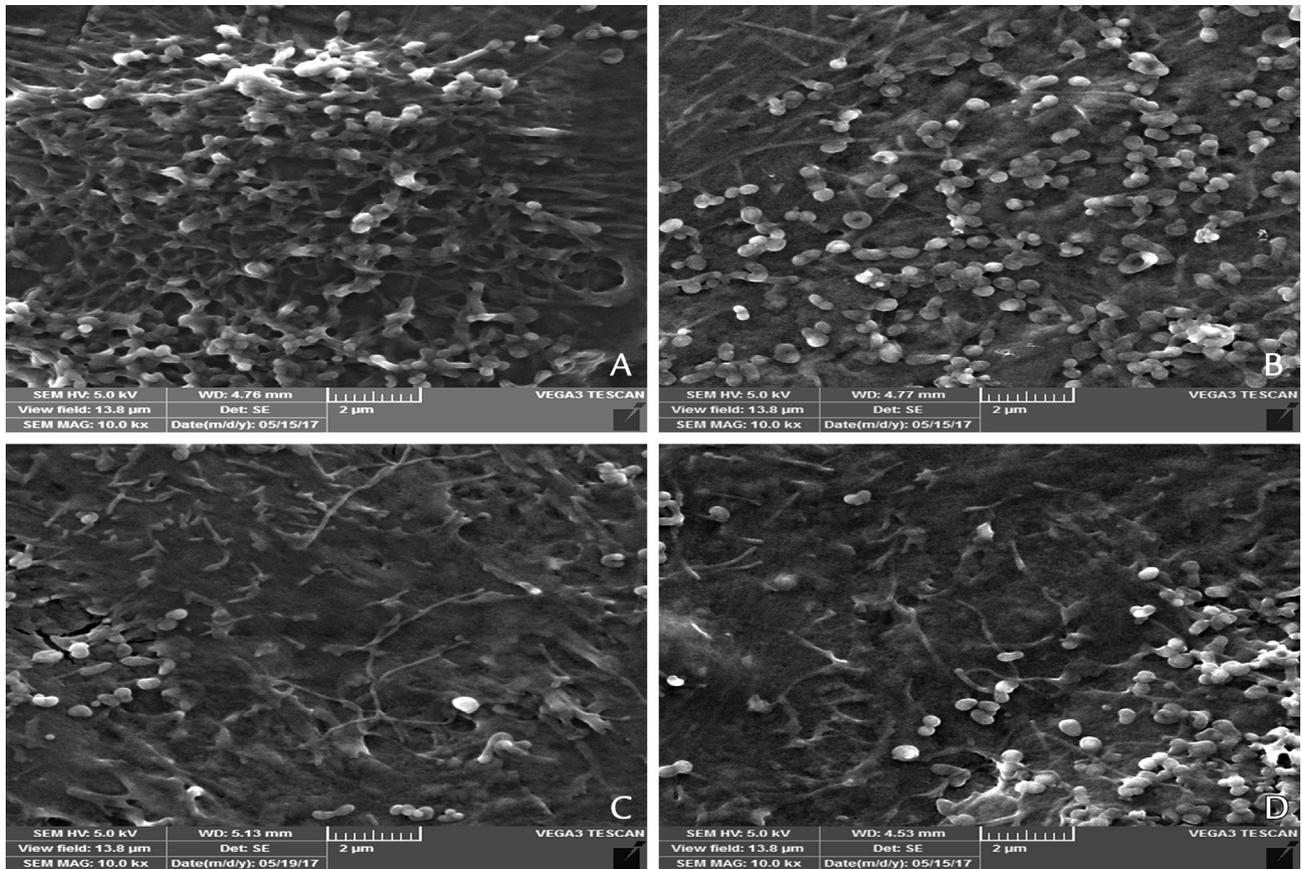


Figure 6. Scanning electron micrographs of Nok-si after 24 hours of adhesion to different substrates. A, ZrO₂ nonaged. B, ZrO₂ aged. C, Ti nonaged. D, Ti aged. Original magnification $\times 10\,000$.

larger than that of the Ti specimens (Table 2). The bacterial count (CFU/mL) is graphically represented in Table 3. The results of the 1-way ANOVA concluded that a significant difference was found in the bacterial growth for Ti (nonaged and aged) in relation to the control ($P < .001$). No differences were found in Ti before and after aging, but ZrO₂ had increased growth of microorganisms after aging. For ZrO₂, a statistically significant difference was found between aged ZrO₂ and the control ($P < .001$).

Figure 9A-D shows images of multispecies biofilms, which adhered after 24 hours to the ZrO₂ and Ti specimens before and after aging. Small bacterial aggregates adhered to all surfaces studied, but on the nonaged and aged Ti surfaces, the microorganisms were sparsely lined with bacteria, which were separated by numerous gaps, exposing parts of the underlying surface of the dental material. However, on ZrO₂, after surface aging, the population of microorganisms was larger, and they were more scattered across the surface.

DISCUSSION

Aging affected the physicochemical properties of the Ti and ZrO₂ implant abutment materials and the adhesion and proliferation of normal oral keratinocytes (Nok-si).

The formation of multispecies biofilms of *F. nucleatum*, *P. gingivalis*, and *S. sanguinis* was reduced after the aging of the materials. Therefore, the null hypothesis was rejected as aging influenced the adhesion of cells and microorganisms on the Ti and ZrO₂ surfaces.

The surface roughness of ZrO₂ did not significantly change after autoclaving, which is consistent with other findings.^{28,29,37} The effects of aging on ZrO₂ may initially occur around the superficial layer in the highest topographical grains, which are also more susceptible to water contact.²⁹ This could also indicate why the aging treatment of autoclaving for 20 hours at 134 °C with 200 kPa of pressure was not significant enough to promote the deleterious effects described by Lugh and Sergio.⁸ The increase in the surface roughness of Ti after aging is probably because of the formation of this layer of Ti oxide. In the case of implant abutments, a 0.2- μm roughness value has been accepted as the minimum average roughness threshold required to maintain the seal between the epithelial cells and the surface, hence the stability of the soft tissue.³⁸

After aging, the hydrophobic characteristic of the ZrO₂ surfaces was maintained, and they presented the highest surface tension value.¹⁸ This result is consistent with that in the study by Han et al.²⁶ After aging, Ti

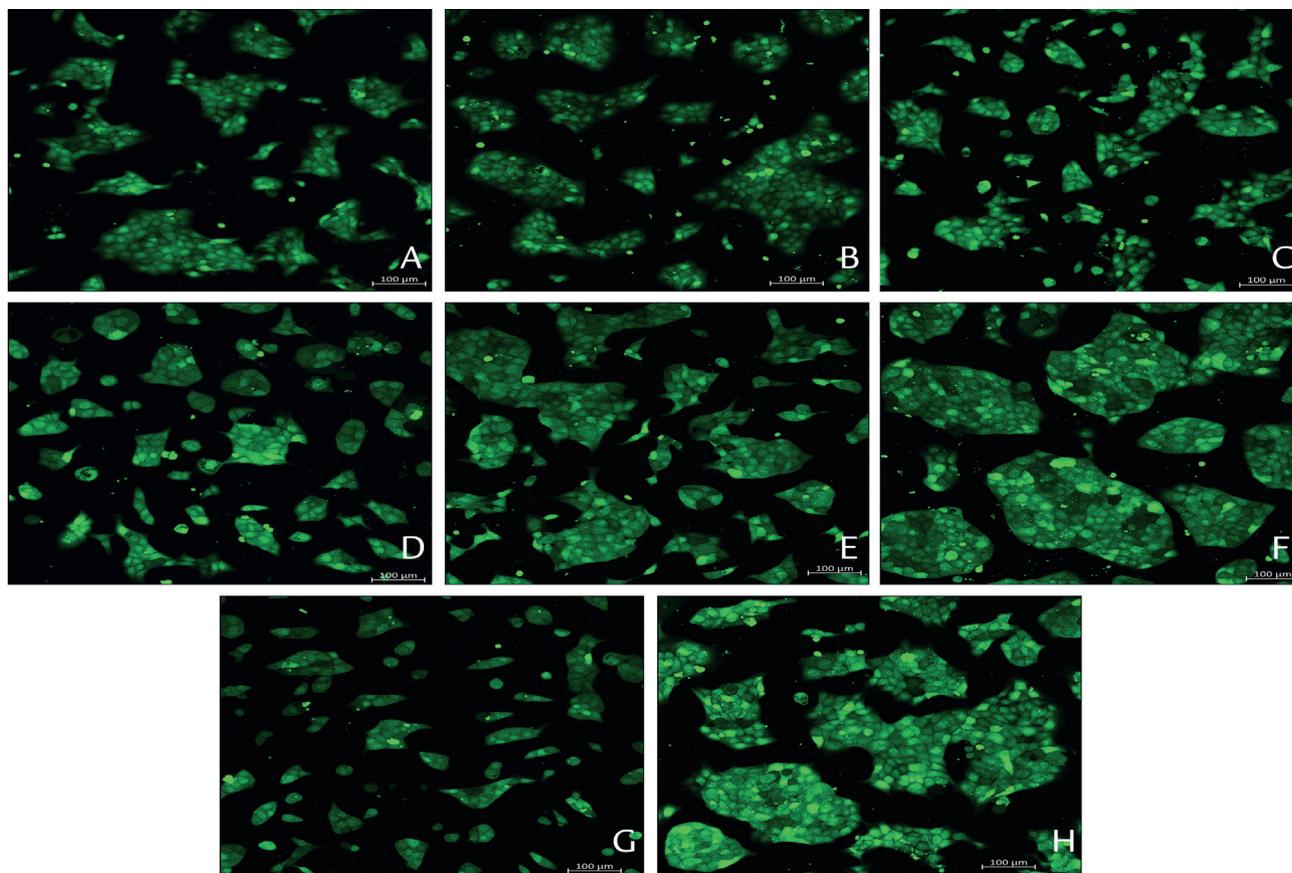


Figure 7. Confocal microscopy showing proliferation of Nok-si after incubation with different substrates for 24 and 48 hours. A, ZrO₂ nonaged at 24 hours. B, ZrO₂ nonaged at 48 hours. C, ZrO₂ aged at 24 hours. D, ZrO₂ aged at 48 hours. E, Ti nonaged at 24 hours. F, Ti nonaged at 48 hours. G, Ti aged at 24 hours. H, Ti aged at 48 hours. Original magnification $\times 10$.

became more hydrophobic and the contact angle increased, thus decreasing the surface interaction with distilled water and characterizing the surface as hydrophobic. The Ti surface maintained its lipophilic characteristic, as evidenced by the decreased contact angle after aging, with liquids of lower material polarity. The SFE value of Ti was similar because of the increased intermolecular interaction forces on the surface. The XRD results on the ZrO₂ surfaces showed a decrease in the tetragonal phase after autoclaving and an increase in the monoclinic phase, a finding consistent with other studies.³⁹⁻⁴² The ability of the tetragonal grains to undergo the phase transformation in response to induced stress can be detrimental, particularly when zirconia is exposed to a humid environment at temperatures ranging from 100 °C to 300 °C.³⁹ This phenomenon is LTD.²⁹ The XRD for Ti showed no change because this surface does not have a crystalline structure.

Regardless of the type of material, the aging process did not affect cell viability or morphology, presumably because the cells adhere to an intermediate layer of deposited extracellular matrix molecules.⁴³ However, a

subtle difference was observed between the materials. The cells adhered on the surface of ZrO₂ presented a more rounded conformation and fewer projections. After aging, the cell proliferation analysis showed an increased number of cells adhered to the Ti surfaces, which maintained their hydrophobic and lipophilic characteristics, suggesting that this characteristic influenced the cells studied.⁴ Similarly, other authors have highlighted SFE as important in determining the adhesion of gingival epithelial cells because a relation between the free surface energy and the conformational state of the adsorbed proteins may influence the biologically active conformation on high surfaces.^{4,43} For cells adhering to ZrO₂, the amount of β -actin dramatically decreased in 48 hours for both the nonaged and aged surfaces. Before and after aging, the cells adhering to the Ti surface had a higher amount of β -actin than the cells adhering to ZrO₂, which suggests better initial establishment of adhesion to this surface and that the adhesion was maintained. This finding may be associated with hydrophobic and lipophilic characteristics of Ti because there is a relationship between SFE and the adsorption of proteins associated

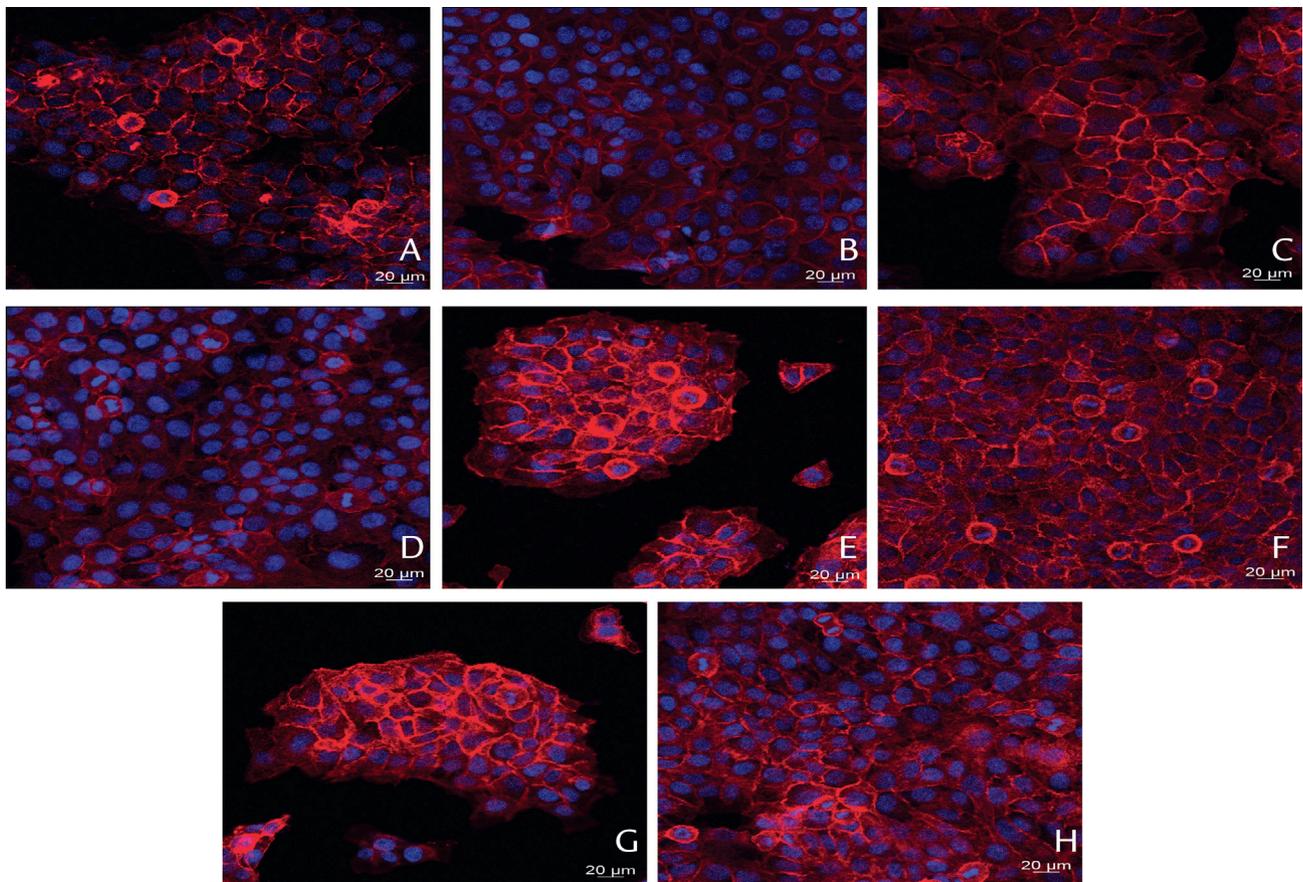


Figure 8. Confocal microscopy showing adhesion of Nok-si to different substrates at 24 and 48 hours. A, ZrO₂ nonaged at 24 hours. B, ZrO₂ nonaged at 48 hours. C, ZrO₂ aged at 24 hours. D, ZrO₂ aged at 48 hours. E, Ti nonaged at 24 hours. F, Ti nonaged at 48 hours. G, Ti aged at 24 hours. H, Ti aged at 48 hours. Original magnification ×20.

Table 2. Quantity of β-actin present in adhered cell surfaces of aged and nonaged titanium and zirconia abutments observed in adhesion test after 24 and 48 hours

Fluorescence Measurement at 405 nm to 561 nm	24 h	48 h
ZrO ₂ /nonaged	22373.329	9742.792
ZrO ₂ /aged	18871.090	8352.457
Ti/nonaged	35589.327	19692.524
Ti/aged	33707.224	20244.763

Ti, titanium; ZrO₂, zirconia.

with cell surface adhesion.^{4,24} Interestingly, this type of cell preferentially adhered to hydrophobic materials, suggesting that, owing to the same chemical nature, the final interaction between the chemical elements of the epithelial cell membrane and the surface is hydrophobic.^{13,19}

In the present study, a higher biofilm adhesion was found to both ZrO₂ surfaces and compared with the Ti surfaces, possibly because of increased SFE.¹¹ These data contrast those reported by Dutra et al,²⁹ in which no difference in biofilm formation was observed after hydrothermal aging simulation. Ti presents low surface polarity and attracts molecules that are of the same

Table 3. Effect of aging on biofilm formation at 72 hours through quantitative measurement of CFU/mL as indicator of bacterial colonization on polystyrene well C+ and Ti (nonaged and aged) surfaces compared with that of ZrO₂ surfaces (nonaged and aged)

Group	Log ₁₀ CFU/mL	
	Mean	SD
ZrO ₂ /nonaged ^a	6.95	0.09
ZrO ₂ /aged ^c	7.23	0.07
Ti/nonaged ^b	6.29	0.11
Ti/aged ^b	6.40	0.12
C+ ^a	7.31	0.13

Ti, titanium; ZrO₂, zirconia. One-way ANOVA used with Tukey post hoc test. Data shown as mean ±SD. Statistically significant differences indicated as P<.05. Same letters represent results with no significant difference.

chemical composition, highly hydrophobic, and lipophilic. *F. nucleatum* and *P. gingivalis* are mainly formed by polysaccharides (molecules with an affinity for hydrophilic surfaces).

More studies are needed to evaluate the behavior of both the cells and microorganisms on the surfaces used as prosthetic components after aging: Ti and ZrO₂. The physical-chemical properties of the surface, which are

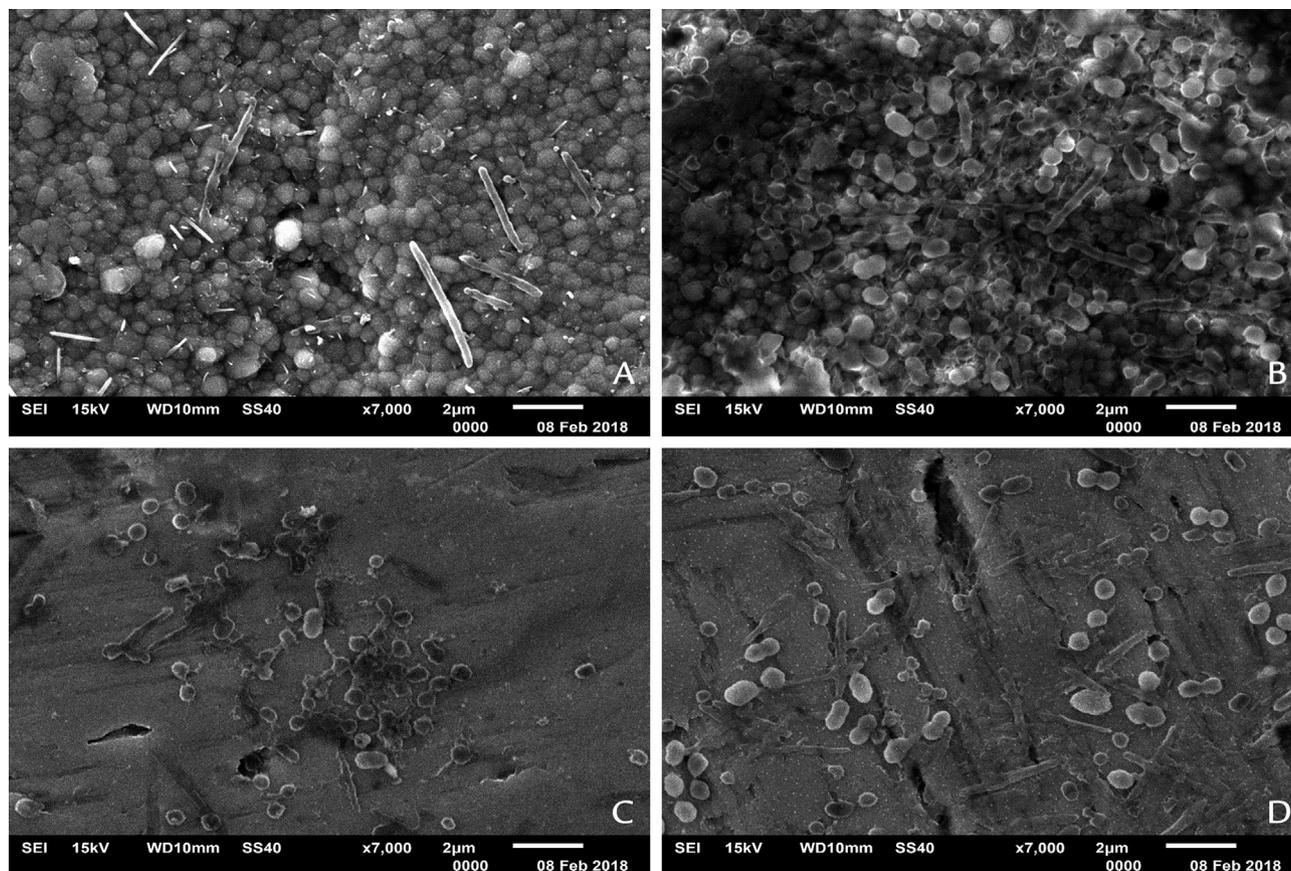


Figure 9. Scanning electron micrographs showing morphology of biofilm after 72 hours of adhesion with different substrates. A, ZrO₂ nonaged. B, ZrO₂ aged. C, Ti nonaged. D, Ti aged. Original magnification $\times 7000$.

significantly altered because of their permanence in the mouth and respective aging processes, influence the extent and manner of cell and bacterial adhesion. However, owing to the complexity of the system, the specific properties are difficult to determine. Moreover, understanding the interaction between these surfaces and the architecture of the peri-implant tissue may be crucial to the long-term success of implant treatment, especially in patients with susceptibility to periodontal diseases. Modifying the surface by changing SFE and chemical composition may be an attempt to reduce bacterial colonization and to influence the interactions between the surface and the microorganisms that contact it.

CONCLUSIONS

Based on the results of this *in vitro* study, the following conclusions were drawn:

- 1 The aging-induced changes in the physicochemical properties of Ti and ZrO₂ influenced the adhesion and proliferation of normal oral keratinocytes.
- 2 The changes in these properties also affected the viability and adhesion of multispecies biofilms of *E. nucleatum*, *S. sanguinis*, and *P. gingivalis*.

- 3 The results indicate that the materials examined are suitable for use as abutments; however, Ti showed better cell adhesion, improved proliferation, and lower biofilm adhesion than ZrO₂.

REFERENCES

1. Lucas TJ, Lawson NC, Janowski GM, Burgess JO. Phase transformation of dental zirconia following artificial aging. *J Biomed Mater Res B Appl Biomater* 2015;103:1519-23.
2. Chevalier J. What future for zirconia as a biomaterial? *Biomaterials* 2006;27:535-43.
3. Chevalier J, Gremillard L, Deville S. Low-temperature degradation of zirconia and implications for biomedical implants. *Annu Rev Mater Res* 2007;37:1-32.
4. Rompen E, Domken O, Degidi M, Farias Pontes AE, Piattelli A. The effect of material characteristics, of surface topography and of implant components and connections on soft tissue integration: a literature review. *Clin Oral Implants Res* 2006;17:55-67.
5. Att W, Yamada M, Ogawa T. Effect of titanium surface characteristics on the behavior and function of oral fibroblasts. *Int J Oral Maxillofac Implants* 2009;24:419-31.
6. Steinemann SG. Titanium – the material of choice? *Periodontol* 1998;17:7-21.
7. Glauser R, Sailer I, Wohlwend A, Studer S, Schibli M, Scharer P. Experimental zirconia abutments for implant-supported single-tooth restorations in esthetically demanding regions: 4-year results of a prospective clinical study. *Int J Prosthodont* 2004;17:285-90.
8. Lugh V, Sergio V. Low temperature degradation “aging” of zirconia: a critical review of the relevant aspects in dentistry. *Dent Mater* 2010;26:807-20.
9. Pereira G, Amaral M, Cesar P, Bottino M, Kleverlaan C, Valandro L. Effect of low-temperature aging on the mechanical behavior of ground Y-TZP. *J Mech Behav Biomed Mater* 2015;45:183-92.

10. Chevalier J, Cales B, Drouin JM. Low-temperature aging of Y-TZP ceramics. *J Am Ceram Soc* 2004;82:2150-4.
11. Quirynen M, Bollen CM. The influence of surface roughness and surface-free energy on supra- and subgingival plaque formation in man: a review of the literature. *J Clin Periodontol* 1995;22:1-14.
12. Lampin M, Warocquier-Clérout R, Legris C, Degrange M, Sigot-Luizard M. Correlation between substratum roughness and wettability, cell adhesion, and cell migration. *J Biomed Mater Res* 1997;36:99-108.
13. de Avila ED, de Molon RS, Palomari Spolidorio DM, de Assis Mollo F Jr. Implications of surface and bulk properties of abutment implants and their degradation in the health of periodontal tissue. *Materials (Basel)* 2013;6:5951-66.
14. de Avila ED, de Molon RS, Vergani CE. The relationship between biofilm and physical-chemical properties of implant abutment materials for successful dental implants. *Materials* 2014;7:3651-62.
15. Popovici J, White CP, Hoelle J, Kinkle BK, Lytle DA. Characterization of the cell surface properties of drinking water pathogens by microbial adhesion to hydrocarbon and electrophoretic mobility measurements. *Colloids Surf B Biointerfaces* 2014;118:126-32.
16. de Avila ED, de Molon RS, Lima BP, Lux R, Shi W, Junior MJ, et al. Impact of physical chemical characteristics of abutment implant surfaces on bacteria adhesion. *J Oral Implantol* 2016;42:153-8.
17. de Avila ED, Avila-Campos MJ, Vergani CE, Spolidório DMP, de Assis Mollo F Jr. Structural and quantitative analysis of a mature anaerobic biofilm on different implant abutment surfaces. *J Prosthet Dent* 2016;115:428-36.
18. de Avila ED, Vergani CE, Mollo Junior FA, Junior MJ, Shi W, Lux R. Effect of titanium and zirconia dental implant abutments on a cultivable polymicrobial saliva community. *J Prosthet Dent* 2017;118:481-7.
19. Rigolin MSM, de Avila ED, Basso FG, Hebling J, de S Costa CA, Mollo Junior FA, et al. Effect of different implant abutment surfaces on OBA-09 epithelial cell adhesion. *Microsc Res Tech* 2017;80:1304-9.
20. Brunot-Gohin C, Duval J-L, Azogui E-E, Jannetta R, Pezron I, Laurent-Maquin D, et al. Soft tissue adhesion of polished versus glazed lithium disilicate ceramic for dental applications. *Dent Mater* 2013;29:e205-12.
21. Buser D, Weber HP, Donath K, Fiorellini JP, Paquette DW, Williams RC. Soft tissue reactions to non-submerged unloaded titanium implants in beagle dogs. *J Periodontol* 1992;63:225-35.
22. Quirynen M, Marechal M, Busscher H, Weerkamp A, Darius P, Steenberghe DV. The influence of surface free energy and surface roughness on early plaque formation. *J Clin Periodontol* 1990;17:138-44.
23. Quirynen M, Bollen CM, Papaioannou W, Van Eldere J, van Steenberghe D. The influence of titanium abutment surface roughness on plaque accumulation and gingivitis: short-term observations. *Int J Oral Maxillofac Implants* 1996;11:169-78.
24. Janssen D, De Palma R, Verlaak S, Heremans P, Dehaen W. Static solvent contact angle measurements, surface free energy and wettability determination of various self-assembled monolayers on silicon dioxide. *Thin Solid Films* 2006;515:1433-8.
25. van Brakel R, Cune MS, van Winkelhoff AJ, de Putter C, Verhoeven JW, van der Reijden W. Early bacterial colonization and soft tissue health around zirconia and titanium abutments: an in vivo study in man. *Clin Oral Implants Res* 2011;22:571-7.
26. Han A, Tsoi JKH, Matinlinna JP, Zhang Y, Chen Z. Effects of different sterilization methods on surface characteristics and biofilm formation on zirconia in vitro. *Dent Mater* 2018;34:272-81.
27. Cotes C, Arata A, Melo RM, Bottino MA, Machado JP, Souza RO. Effects of aging procedures on the topographic surface, structural stability, and mechanical strength of a ZrO₂-based dental ceramic. *Dent Mater* 2014;30:e396-404.
28. Park JH, Olivares-Navarrete R, Baier RE, Meyer AE, Tannenbaum R, Boyan BD, et al. Effect of cleaning and sterilization on titanium implant surface properties and cellular response. *Acta Biomater* 2012;8:1966-75.
29. Dutra D, Pereira G, Kantorski KZ, Exterkate R, Kleverlaan CJ, Valandro LF, et al. Grinding with diamond burs and hydrothermal aging of a Y-TZP material: effect on the material surface characteristics and bacterial adhesion. *Oper Dent* 2017;42:669-78.
30. Castilho RM, Squarize CH, Leelahavanichkul K, Zheng Y, Bugge T, Gutkind JS. Rac1 is required for epithelial stem cell function during dermal and oral mucosal wound healing but not for tissue homeostasis in mice. *PLoS One* 2010;5:e10503.
31. Sánchez M, Fernández E, Llama-Palacios A, Figuero E, Herrera D, Sanz M. Response to antiseptic agents of periodontal pathogens in vitro biofilms on titanium and zirconium surfaces. *Dent Mater* 2017;33:446-53.
32. Rimondini L, Cerroni L, Carrassi A, Torriceni P. Bacterial colonization of zirconia ceramic surfaces: an in vitro and in vivo study. *Int J Oral Maxillofac Implants* 2002;17:793-8.
33. Lee BC, Jung GY, Kim DJ, Han JS. Initial bacterial adhesion on resin, titanium and zirconia in vitro. *J Adv Prosthodont* 2011;3:81-4.
34. Nagayama M, Sato M, Yamaguchi R, Tokuda C, Takeuchi H. Evaluation of co-aggregation among *Streptococcus mitis*, *Fusobacterium nucleatum* and *Porphyromonas gingivalis*. *Lett Appl Microbiol* 2001;33:122-5.
35. Pier-Francesco A, Adams RJ, Waters MGJ, Williams DW. Titanium surface modification and its effect on the adherence of *Porphyromonas gingivalis*: an in vitro study. *Clin Oral Implants Res* 2006;17:633-7.
36. Bürgers R, Gerlach T, Hahnel S, Schwarz F, Handel G, Gosau M. In vivo and in vitro biofilm formation on two different titanium implant surfaces. *Clin Oral Implants Res* 2010;21:156-64.
37. Muñoz EM, Longhini D, Antonio SG, Adabo GL. The effects of mechanical and hydrothermal aging on microstructure and biaxial flexural strength of an anterior and a posterior monolithic zirconia. *J Dent* 2017;63:94-102.
38. Mustafa K, Wennerberg A, Arvidson K, Messelt EB, Haag P, Karlsson S. Influence of modifying and veneering the surface of ceramic abutments on cellular attachment and proliferation. *Clin Oral Implants Res* 2008;19:1178-87.
39. Zhang Y, Pajares A, Lawn BR. Fatigue and damage tolerance of Y-TZP ceramics in layered biomechanical systems. *J Biomed Mater Res B Appl Biomater* 2004;71:166-71.
40. Roy ME, Whiteside LA, Katerberg BJ, Steiger JA. Phase transformation, roughness, and microhardness of artificially aged yttria- and magnesia-stabilized zirconia femoral heads. *J Biomed Mater Res A* 2007;83:1096-102.
41. Flinn BD, Raigrodski AJ, Singh A, Mancl LA. Effect of hydrothermal degradation on three types of zirconias for dental application. *J Prosthet Dent* 2014;112:1377-84.
42. Basílio MA, Cardoso KV, Antonio SG, Rizkalla AS, Santos Junior GC, Arioli Filho JN. Effects of artificial aging conditions on yttria-stabilized zirconia implant abutments. *J Prosthet Dent* 2016;116:277-85.
43. Jansen JA, Den Braber ET, Walboomers XF, De Ruijter JE. Soft tissue and epithelial models. *Adv Dent Res* 1999;13:57-66.

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Acknowledgments

The authors would like to thank Professor Dr Miguel Jafelici Jr who allowed the use of the goniometry equipment (Institute of Chemistry-UNESP); Professor Dr Carlos Rossa Junior (Araraquara Dental School-UNESP) for donating the Noksi cells; Conexão Sistemas de Próteses Company (Ltda, SP, Brazil) for donating the disk-shaped specimens of both materials: pure commercially available titanium and zirconia stabilized with yttrium; Professor Carina Domaneschi by sending the disks after sterilization; IPEN (Institute of Nuclear Energy Research, SP, Brazil) for sterilization by using gamma irradiation of the discs; Danubia Gava for X-ray diffraction analysis; Franca University where the scanning electron microscopy analysis of the cells and the adhesion of the microorganisms were carried out; Maria Anita Vasconcelos Ambrosio for adhesion of the microorganisms; and Professor Dr Erica Dorigatti de Ávila for performing part of the statistical analysis, and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Capes for the support in study material and scholarship.

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<https://doi.org/10.1016/j.prosdent.2019.08.027>