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Ultraviolet light-treated zirconia with different roughness affects function of human gingival fibroblasts *in vitro*: The potential surface modification developed from implant to abutment

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Abstract: Objectives: To evaluate whether ultraviolet (UV) light irradiation of smooth and rough zirconia disks enhances its biocompatibility with human gingival fibroblasts (HGFs).

Materials and Methods: Zirconia disks were divided into four groups: smooth control (S-C), smooth with UV light treatment (S-UV), rough control (R-C), and rough with UV light treatment (R-UV). The surface morphology and wettability were analyzed, and X-ray photoelectron spectroscopy was carried out. The cultured HGFs' adhesive density, morphology, proliferation, and collagen synthesis were measured on different time points from 3 h to 7 days.

Results: After 24-h UV light treatment, contact angles decreased from 51.98° (S-C) and 63.87° (R-C) to 33.76° (S-UV) and 36.15° (R-UV). X-ray photoelectron spectroscopy analysis showed that surface C/O ratio reduced from 1.86 and 2.39 to 1.33 and 1.19. After UV light treatment, cells initial spreading areas and perimeters were doubled on S-UV disks and nearly tripled on R-UV disks. Three hours cell adhesion was enhanced on S-UV disks, and 24-h cell density was increased on R-UV. Cell proliferation of 48 or 72 h was all significantly changed on UV-treated disks. Cells on R-UV also released highest level of Col-1 after 3 and 7 days, whereas those on S-UV produced less levels compared with control.

Conclusion: Our study demonstrated for the first time that UV light treatment on rough ($Ra = 0.19 \pm 0.03$ μm) zirconia had a positive effect on behavior of HGFs, including 24-h cell adhesion, proliferation, and collagen release. The effect varied with surface roughness and time point. Surface morphology and hydrophilicity, as two interacted factors, both are governed on HGFs behavior. © 2014 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater, 103B: 116–124, 2015.

Key Words: UV light bioactivation, surface roughness, zirconia, implant interface, fibroblasts

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INTRODUCTION

In the past 40 years, dental implant technique has achieved a great success in clinical work. The rapid and sustained osseointegration ensures the long-term success. However, several *in vitro* and animal studies have demonstrated that the interface between implant abutment and peri-implant soft tissue is also a vital factor for implant success.[1] A soft tissue barrier around abutment protects the underlying bone and maintains the natural shape of gingiva.[2,3] The formation and maintenance of soft tissue barrier mainly depends on the response of peri-implant soft tissues to implant materials.[4–7] For one-piece implants (tissue level), the critical area is the neck portion of the implants. For two-piece implants (bone level), the critical area is the material and surface of the abutments. Therefore, more and more interest has focused on the modification of implant surfaces, to improve the soft tissue sealing around them.

Just like the osteoblasts playing an important role in osseointegration, human gingival fibroblasts (HGFs) help to maintain the soft tissue barrier. It is well established that HGFs are major cells in soft tissues, and a large number of fibroblasts are present in the narrow zone of the connective tissue immediately adjacent to the abutment.[8] That is why in many *in vitro* studies HGFs were chosen to represent the peri-implant soft tissues and evaluate the biocompatibility of implant and abutment materials.

As regard to abutment materials, titanium is popular both for implant and abutment,[9] and it has achieved a great

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success in long-term clinical practice. In addition, ceramic materials like yttrium-stabilized zirconia (TZP) have been recently introduced as an alternative material for implant abutments. Its tooth-like color offers great esthetic benefits. In *in vitro* studies, zirconia disks showed lower bacterial deposition[10] and greater biocompatibility compared with titanium.[11] Therefore, zirconia has been recommended to be used in esthetic zone.[12]

Surface topography and physicochemistry are two vital factors that affect cells response to material interfaces. Surface roughness of zirconia has a marked effect on soft tissues, which has been observed by many researchers.[13] It is recognized that a certain threshold of roughness (Ra = 0.2 μm) is necessary to achieve a stable soft tissue seal.[11] However, Mustafa et al.[14] demonstrated that increasing surface roughness to 0.34 μm did not inhibit fibroblast attachment. It indicates that, under a certain threshold of roughness, larger surface area achieved by milling process may be better for the cells' adhesion and proliferation.

Physicochemical properties like hydrophilicity have been proved to impact cell adhesion and proliferation, by other researchers.[15–18] It is recognized that high surface wettability may enhance cell function. Ultraviolet (UV) light irradiation on zirconia has been proved to be one of the means of modifying its wettability.[17,19–21] It has been reported that UV-treated zirconia enhanced the behavior of osteoblasts,[17] which benefits its application on implant surface modification to get greater osseointegration. But so far, few studies focused on its biological properties on soft tissues.[22] Furthermore, the changing wettability was more remarkable on rough surface than on smooth surface.[23] No study reports the different effect of UV-treated zirconia with smooth and rough surface on fibroblast behavior. Whether UV light treatment can be expanded to abutment surface is uncharted.

For the present study, the objective was to investigate the behavior of HGFs cultured on zirconia disks with different surface roughness, and to determine whether UV light treatment could enhance zirconia's bioactivity on HGFs. The null hypothesis was: after the zirconia disk surfaces being UV light-treated, the response of cells maintains the same, neither for smooth or rough disks. HGF cells' adhesion, proliferation, and collagen release were measured.

MATERIALS AND METHODS

Zirconia samples and UV light treatment

Zirconia disks (Zenostar, Wieland Dental, Germany) (20 mm in diameter and 2 mm thick) were first obtained using a cutting machine. Smooth specimens were polished with silicon carbide papers in decreasing grain sizes (from 150 to 600 Cw). Rough specimens were prepared by air abrasion with aluminum oxide (Al_2O_3) particles (25 μm) from a distance of about 20 mm at 0.2 MPa air pressure. After that, some specimens were treated with UV light for 24 h under ambient conditions using a 10-W bactericidal lamp (Philip, Eindhoven, the Netherlands), and the measured intensity was 17 mW/cm^2 ($\lambda = 250 \pm 20$ nm). The rest of them were stored at dark and dry condition. The disks were divided into four groups: group S-C, zirconia disks were polished into smooth surface, without UV light treatment; group S-UV, polished and have UV light treatment for 24 h; group R-C, zirconia disks were prepared by air abrasion, without UV light treatment; and group R-UV, after air abrasion and have UV light treatment for 24 h. Prior to the cell culture, all specimens were ultrasonically cleaned for 15 min, with absolute ethanol and distilled water, and were disinfected for 30 min with 75% alcohol.

Surface analysis

The surfaces were first examined by scanning electron microscopy (SEM) (S-4800; Hitachi, Tokyo, Japan) to determine their surface morphology. The surface roughness was evaluated using a profilometer (Mitutoyo Surftest 401 Analyzer Series 200; Mitutoyo Corp., Minatoku, Japan). The arithmetical mean surface roughness (Ra in μm) was determined with a cut-off value of 0.8 mm, measurement length of 4 mm. Five measurements at different locations were recorded for each disk, and the average of three disks was used to obtain the Ra value of each group. The surface wettability of zirconia samples was examined by the contact angle of 1 μL H_2O droplet using a contact angle meter (SL200; USA Kino Industry). To determine their crystalline structure, the disks were examined by X-ray diffraction (D8 advance; Bruker AXS, Karlsruhe, Germany). Electron spectroscopy for chemical analysis was used to evaluate the composition of the outermost surface. It was performed by X-ray photoelectron spectroscopy (XPS) (ESCALAB 250; ThermoFisher Scientific, Waltham, MA), to evaluate the intensity of C1s and O1s. The binding energy of each spectrum was calibrated with the C1s (285.0 eV).

Cell culture

HGFs were grown from gingival biopsy specimens obtained during periodontal surgery on a periodontally healthy human subject with the advanced approval from Institutional Review Board of Peking University School of Stomatology. After getting gingival specimens, phosphate-buffered saline containing 3% antibiotic–antimycotic (Gibco BRL Co., Gaithersburg, MD) was used to wash them for three times. Then minced pieces of tissue were explanted to cell culture dishes (10 cm) containing 10 mL of Dulbecco's modified Eagle medium (Gibco BRL Co.) supplemented with 10% fetal bovine serum (Gibco BRL Co.) and 1% antibiotic–antimycotic solution. Cells were incubated at 37°C in a humidified atmosphere and 5% carbon dioxide. The culture medium was routinely changed every 2 days. Cells from passages four to seven were used for the experiments.

Cell adhesion and proliferation assays (CCK-8 analysis)

Zirconia disks were placed in a 24-well plate. Then, 2×10^5 HGFs were seeded on each disk. The adhesion of HGFs was evaluated after 3 and 24 h of incubation, by measuring the density of the cells attached to disks with a cell counting kit-8 (CCK-8) test assay (Dojindo, Kyushu, Japan). After 48 and 72 h, the proliferation of cells was measured. At each time point, the cells were washed with phosphate-

buffered saline for three times, and CCK-8 solution (100 μL CCK-8 per mL of cell culture medium) was added. After incubation at 37°C for 2 h, optical density of the solution was measured on a spectrophotometer (ELX808, BioTek, Winooski, VT), with wavelengths at 450 nm. Experiments of all four groups were repeated in triplicates, and each group had five disks.

Cell morphology and morphometry

To observe cell attachment and spreading on the different groups of disks, 5×10^4 HGFs were added and cultured for 3 h. Then HGFs on disks were fixed in 95% alcohol for 30 min and stained using fluorescein isothiocyanate–phalloidin (actin filament green color; Sigma, St. Louis, MO) and 4′,6-diamidino-2-phenylindole (nuclei blue color; Roche, Basler, Switzerland). Fluorescence images were photographed using a fluorescence microscope (Eclipse-80i, Nikon, Japan). Five random images were obtained of each group of disks, and experiments of all four groups were repeated in triplicates. About 25 single cells were used to assess cell morphology. Cell area and perimeter were quantified using an image analyzer (ImageJ, version 2, NIH).

Matrix formation of HGF cells

With a human collagen type 1 (Col-1) enzyme-linked immunosorbent assay kit (Bluegene Biotech, Shanghai, China), the amount of Col released into the cell culture medium was determined. After 3 and 7 days of culture, 50 μL of cell culture medium was added to each well of a 96-well plate with 100 μL of conjugate, and incubated at 37°C for 1 h. After that, each well was washed with a washing buffer five times. After washing, 50 μL of substrate A and 50 μL of substrate B solution were added to each well and incubated in the dark at 37°C for 15 min. Finally, 50 μL of stop solution was added to each well, and the optical density was measured at 450 nm on a spectrophotometer (ELX808, BioTek). Total protein of cells on each disks was also measured using BCA Protein Assay Kit (Thermo scientific, Rockford, IL). The final collagen concentration was normalized on total protein. Experiments of all four groups were repeated in triplicates, and each group had four disks.

Statistical analysis

All the results were expressed as means and standard deviations and tested for statistical significance with one-way analysis of variance. A significance level of 0.05 was used in all statistical comparisons. *Post hoc* analysis using the Tukey method was performed to detect pairs of groups with statistically significant differences. All statistical analyses were performed with SPSS software 17.0 (SPSS, IBM).

RESULTS

Surface morphology and photogenerated hydrophilicity of zirconia

With macroscopic observation, little difference in morphology could be found among all four groups [Figure 1(a–d)]. The average roughness (Ra) of smooth groups was 0.05 ± 0.01 μm, and Ra of rough groups was 0.19 ± 0.03 μm. SEM images are presented in Figure 1. The smooth specimens showed a relatively smooth morphology with some typical traces from grinding process, whereas rough disks displayed an overall heterogeneous distribution of micropores, which were caused by blasting. No significant difference was found after UV light irradiation. The contact angles of smooth surfaces were smaller than that of rough surfaces. They both decreased significantly after UV light treatment, from 51.98° and 63.87° for smooth disk and rough disk, respectively, to 33.76° and 36.15°, which displayed more hydrophilic surfaces than before (Figure 2).

Crystalline and XPS analysis of zirconia

XRD analysis presented that this specimen fits the properties of zirconium yttrium oxide (Figure 3). The peak of 30.5° can only be detected for the tetragonal phase of ZrO_2, which is the major constituent of TZP. The XPS analysis of zirconia specimens showed peaks of C1s, O1s, Ca2p3, N1s, Y3d, and Zr3d (Figure 4). XPS analysis showed reduction of C1s peak after UV light treatment (Figure 5). After UV light irradiation, the atomic percentage of carbon (at %) on the outermost surface of smooth and rough zirconia disks were both decreased, so was the surface C/O ratio (Table I).

Morphology of the HGFs

After 3 h of culture, the HGFs had attached to all four surfaces, with a flat morphology. The morphology of fibroblasts cultured on four disks is shown on Figure 6. Fibroblasts on control groups were round with little cellular processes, whereas HGFs on UV-treated disks were clearly larger and spreading to a greater extent. After calculating the area of HGFs, significant differences were shown between UV-treated groups and control groups ($p < 0.05$; Figure 7). Cells on R-UV had the largest area. The perimeters of cells on S-UV and R-UV were similar, both larger than cells on S-C and R-C ($p < 0.05$; Figure 8).

Cell attachment and proliferation

The optical density of attached HGFs of four groups at different time points is shown in Figure 6 and Table II. After 3 h of culture, S-UV had the highest OD, whereas after 24 h the OD of R-UV was higher than that of control [$p < 0.05$; Figure 6(a)]. Control groups had no significant difference in their ODs regardless of smooth or rough surfaces. It showed an equal and even greater ability of cell adhesion on UV-treated zirconia compared with control.

After 48 and 72 h of culture, OD values of UV-treated surfaces were consistently greater than that of control [$p < 0.05$; Figure 6(b)], both for smooth and rough groups. R-UV had the highest OD after 72 h culture. It demonstrated an enhanced proliferation of HGFs on UV-treated surfaces (Figures 9 and 10).

Collagen release

The collagen released into the cell culture medium was measured after 3 and 7 days (Figure 11). At 3 days, the level of Col in R-UV group was the highest. R-C and S-C had no difference ($p > 0.05$), whereas S-UV group had the least

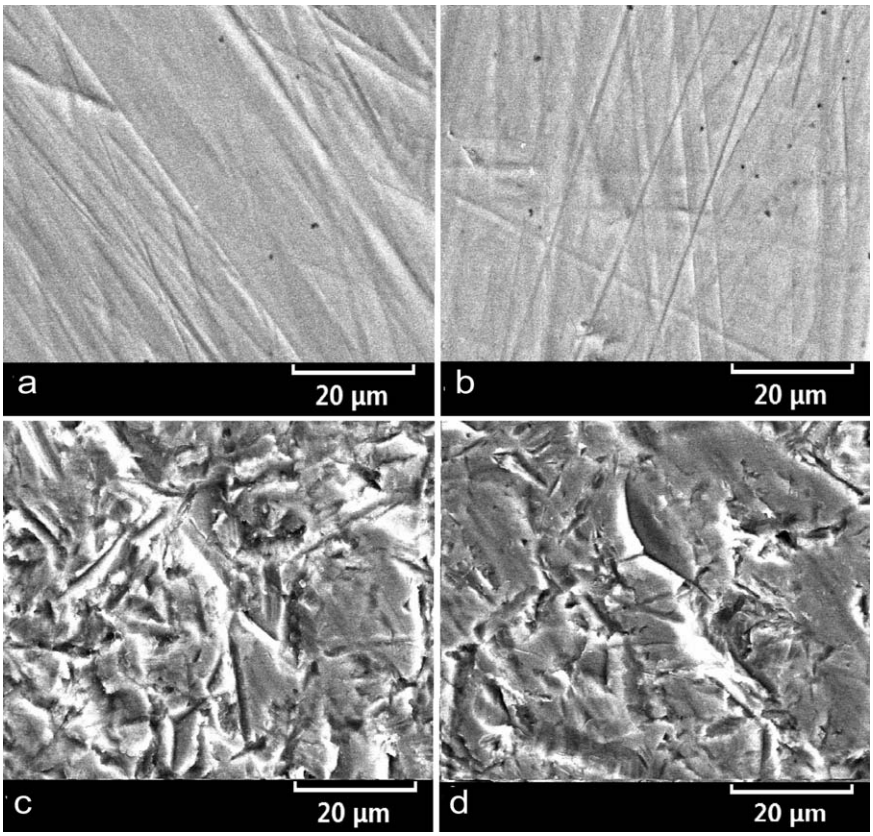

FIGURE 1. Surface morphology of four groups of zirconia as SEM images: (a) S-C surface, (b) S-UV surface, (c) R-C surface, and (d) R-UV surface.

Col release. At 7 days, the highest and lowest Col level was also in R-UV and S-UV group ($p < 0.05$), respectively. As a result, UV-treatment on rough groups advanced the ability of HGFs to release Col, but on smooth surfaces it had the reverse effect.

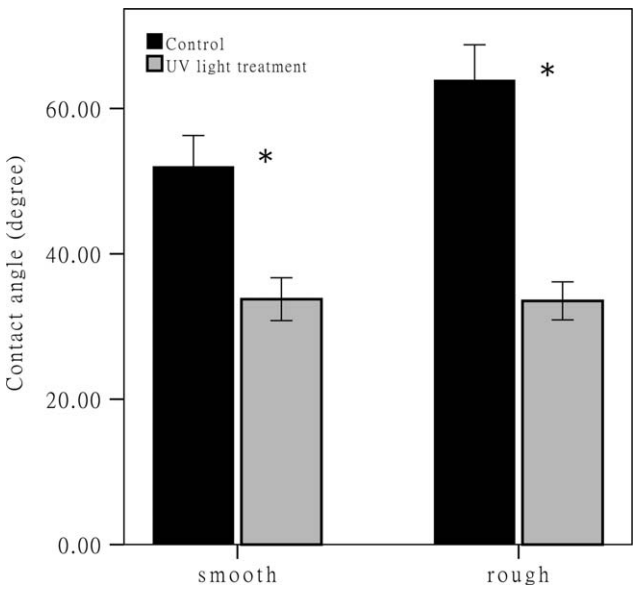

FIGURE 2. After UV light treatment for 24 h, the surface contact angles significantly decreased. Data are shown as mean ± SD (n = 15); *p < 0.05.

DISCUSSION

TZP has already been widely used as implant abutment, which benefits from its esthetic property and great biocompatibility. To improve the ability of peri-implant soft tissues sealing around zirconia, modification of surface is necessary. UV light treatment has been proven being able to change

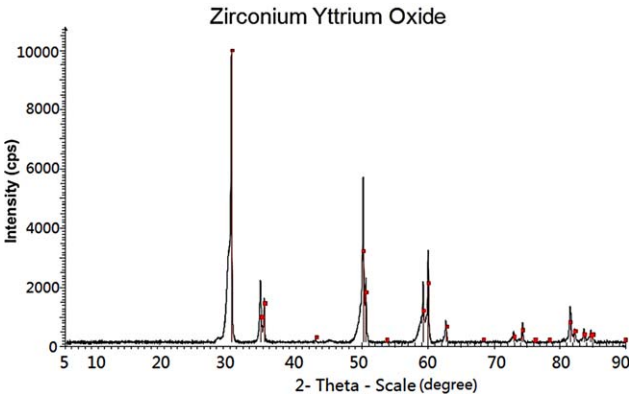

FIGURE 3. X-ray diffraction spectrum of zirconia disks. Its accordance with zirconium yttrium oxide. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

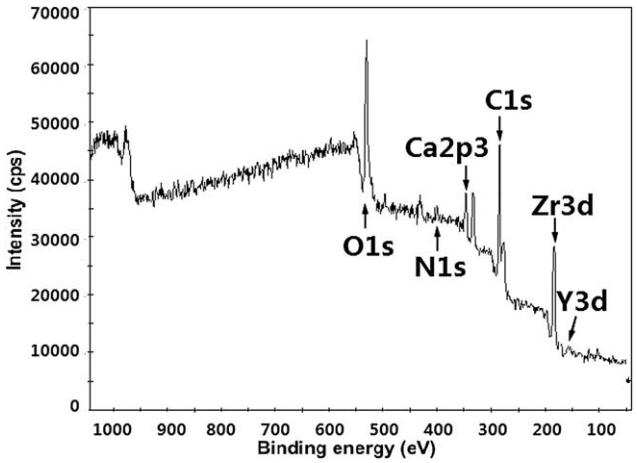

FIGURE 4. XPS spectrum of zirconia disks.

the hydrohphilic property of zirconia-based implant materials, from hydrophobic to hydrophilic (contact angle $< 30^{\circ}$), which is capable to enhance the function of osteoblasts.[17] This effect may also have similar influence on HGFs, and UV light treatment can be expanded from modification of implant surfaces to abutment surfaces. On the other hand, surface roughness is the basic characteristic of surface morphology, which is another proven factor that might affect the adhesion and proliferation of HGFs.[6,11]

In this study, we focused on these two key factors: surface roughness and surface wettability. Different reaction of HGFs was observed on UV light-treated surfaces compared with control, and this UV light effect varied for different roughnesses. The results partly rejected the null hypothesis

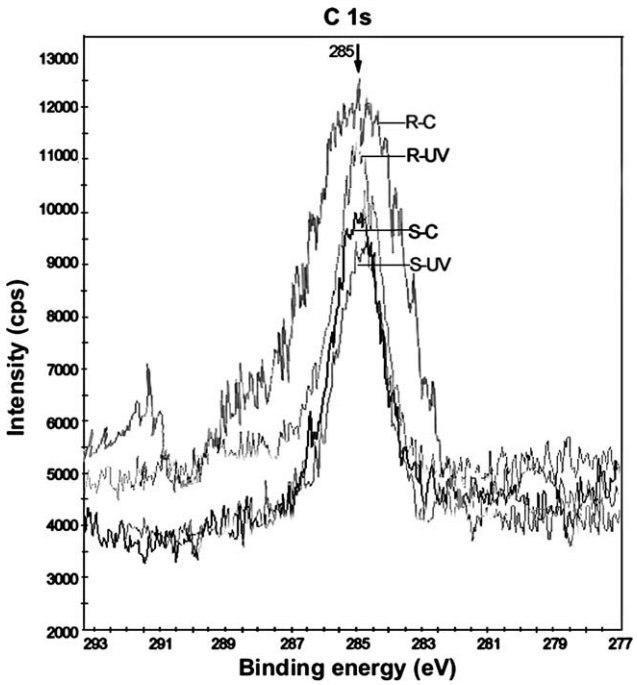

FIGURE 5. The XPS C1s peak of four surfaces. After UV light treatment the peak decreased.

TABLE I. Atomic Percentage of C1s and O1s on Four Surfaces

	C1s (at %)	O1s (at %)	C/O Ratio
S-C surface	55.29	29.8	1.86
S-UV surface	43.03	32.32	1.33
R-C surface	63.42	26.51	2.39
R-UV surface	42.25	35.32	1.19

and demonstrated that UV light treatment on relatively smooth ($Ra < 0.2$ μm) TZP surfaces indeed affected the function of HGFs on them.

The HGFs were separated from human healthy gingival biopsy specimens, and cells from passages four to seven were used in all experiments to mimic real healing process in human body. *In vivo*, during the healing process after implant surgery, the proliferation of connective tissues occurs through the action of fibroblasts and with the help of collagen which the HGFs produce.[24] So not only the adhesion and proliferation of HGFs that concerned but also the releasing collagen which represent cellular maturation was measured in our study.

Surface morphology and topography of four groups of zirconia disks were first examined. Significant differences were found between smooth and rough groups through SEM and surface roughness measurements. Although the optimal surface roughness of implant abutments to promote sustained soft tissue seal has yet to be determined, the limits of threshold roughness ($Ra = 0.2$ μm) had become a consensus to insure the stable soft tissue sealing on the surface. It makes sure *in vivo* study the plaque formation on the surface is acceptable as well.[25] Cochran et al.[13] have reported that a decrease in the amount of attached gingival fibroblast cells was found on the rough surfaces compared with those on smooth surfaces. Other investigations have also reported that smooth surfaces favor human oral fibroblast attachment,[26,27] whereas rough surfaces favor osteoblast adhesion and growth.[28–30] Our study demonstrated that compared with rough surface ($Ra = 0.19$ μm), smooth surface ($Ra = 0.05$ μm) only favored HGFs 48 h proliferation and collagen release after 3 and 7 days. With regard to adhesive density and morphology, surfaces with diverse roughness had no significant difference.

After 24 h of UV light treatment, surface morphology of zirconia maintained the same, whereas the contact angles decreased from more than 50° to more than 30° (Figure 2). The changes in wettability in both smooth and rough groups were not as much as hypothesized, may be because the surfaces were much smoother than that in other studies.[17] But for rough surface, the UV effects were more remarkable, which was in accordance with reports before.[21] It may be due to the larger surface area of rough surface for absorbing UV light.[31] The XPS analysis also showed that for rough disk, after UV light treatment, the decrease in C1s peak and the reduction in C/O were both more conspicuous than that for smooth disk (Table I).

The remarkable change in hydrophilicity has significant influence on HGFs as well. For cell adhesion, UV light

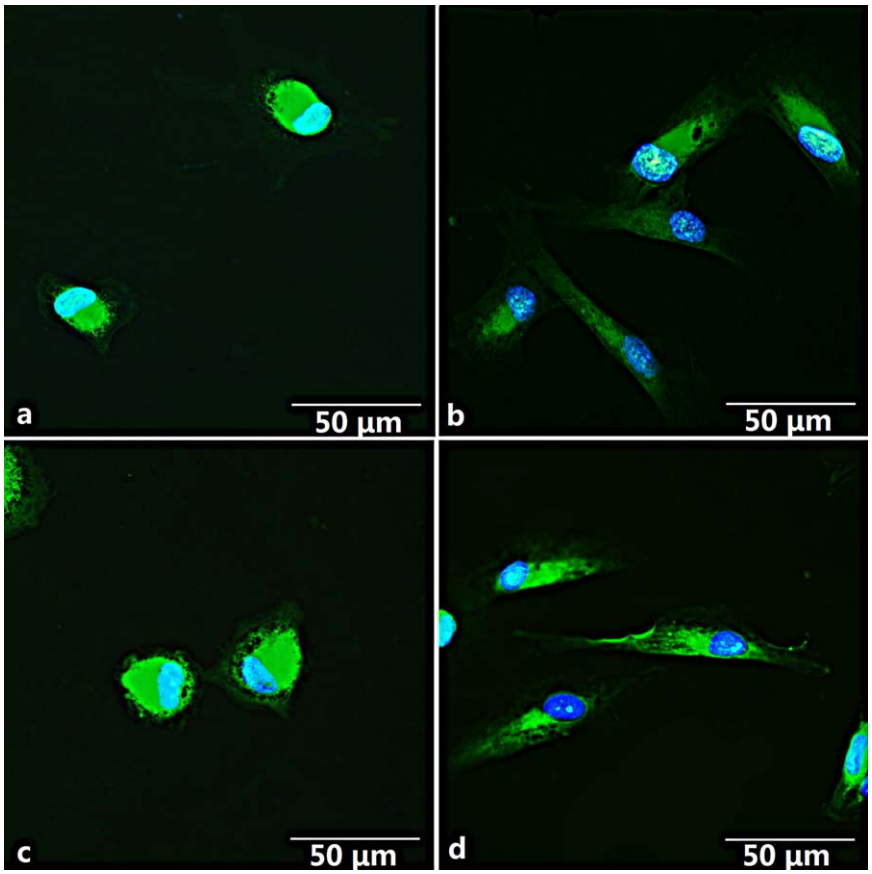

FIGURE 6. Morphology of HGFs on four disks, after cultured for 3 h. (a) S-C surface, (b) S-UV surface, (c) R-C surface, and (d) R-UV surface. The HGFs were stained by fluorescein isothiocyanate–phalloidin (green) and 4′,6-diamidino-2-phenylindole. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

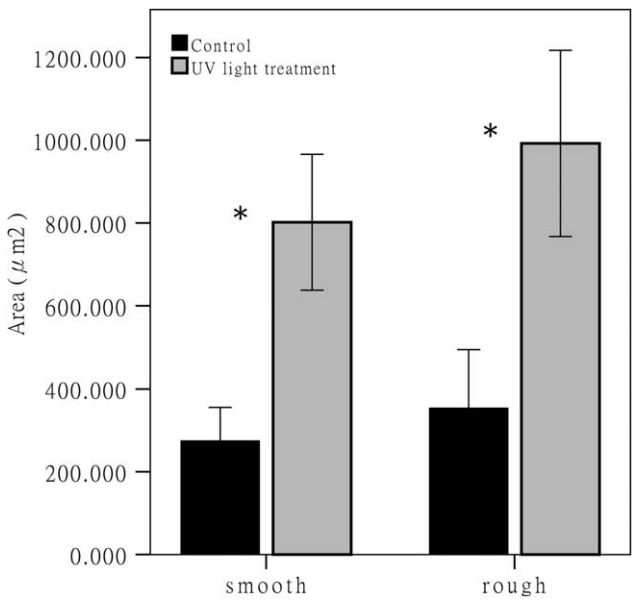

FIGURE 7. Cell morphometric measurements. The spreading areas of cells on four surfaces. Data are shown as mean ± SD (n = 25); *p < 0.05.

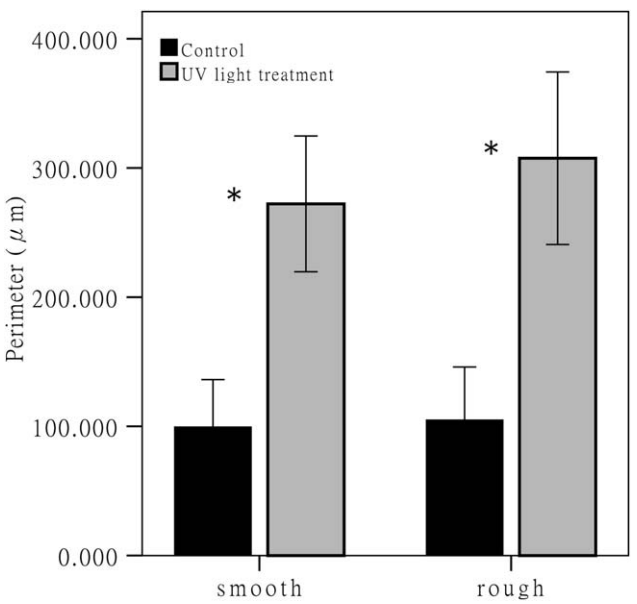

FIGURE 8. Cell morphometric measurements. The perimeters of cells on four surfaces. Data are shown as mean ± SD (n = 25); *p < 0.05.

TABLE II. Cell Adhesion and Proliferation at Different Time Points

		Cell Density (OD, CCK-8) (Mean ± SD)			
Group	N	3 h	24 h	48 h	72 h
S-C	15	0.270 ± 0.076	1.007 ± 0.041	0.920 ± 0.047	0.92076 ± 0.047
S-UV	15	0.375 ± 0.056	1.151 ± 0.024	1.249 ± 0.130	1.24925 ± 0.130
R-C	15	0.328 ± 0.007	1.544 ± 0.154	0.661 ± 0.180	0.66133 ± 0.180
R-UV	15	0.331 ± 0.015	2.005 ± 0.062	1.113 ± 0.386	1.11371 ± 0.386

treatment largely advanced the cell spreading areas and perimeters for both smooth and rough groups. However, the cells density of groups R-UV and S-UV changed differently at 3 and 24 h. For rough zirconia, it seemed that UV light enhanced the adhesion of HGFs in 24 h. But for smooth disks, UV light seemed only good for initial (3 h) cell adhesion. With respect to proliferation, HGFs proliferated significantly faster after UV light treatment on both smooth and rough zirconia. Col-1 is cellular matrix produced by HGFs, and is the main composition of collagen in soft tissues. Our study normalized the concentrations of collagen on total cellular protein on each sample, which eliminated the effects of different cellular quantities. The ability of releasing Col changed differently depends on surface roughness. Cells on R-UV produced a lot more Col than those on R-C for both time points. For smooth disks, on the contrary, UV light even decreased the releasing collagen after 3 and 7 days. All this leads up to R-UV becoming the most favorable surface for HGFs' adhesion, proliferation, and releasing collagen. UV light treatment had better effect on rough zirconia when concerning HGFs biological behaviors.

Titanium dioxide was discovered as UV-photocatalytic material in 1997.[32] ZrO_2 is another photocatalyst material, which corresponds to wavelength of approximately 250 nm.[33] This study demonstrated that UV-C light treatment was still effective on relatively smooth ($Ra < 0.2$ μm) TZP to remove hydrocarbon on surface, and the effect on HGFs was positive. UV light irradiation of zirconia apparently removed the surface carbon to increase the surface wettability, without changing surface topography. For rough surface, this effect seemed greater than smooth surface. It may explain the greater extent of enhanced cell function on R-UV disks than on S-UV disks, including cell adhesion, cell proliferation, and releasing Col. But as seen in current studies, it seems difficult to make a direct link between surface wettability and biocompatibility. For smooth zirconia, after UV light treatment, there was obvious change in contact angle and C/O ratio, but the density of adhesive cells and releasing Col-1 were lower than that of control. Beyond this, the contact angles of S-UV and R-UV were nearly the same, but the behavior of HGFs on these two specimens were different. It may illustrate that surface morphology and hydrophilicity are two independent factors, which affect HGFs on material interfaces as a result of their interaction. Besides all, many other vital factors should be noted when considering soft tissue sealing around abutments.

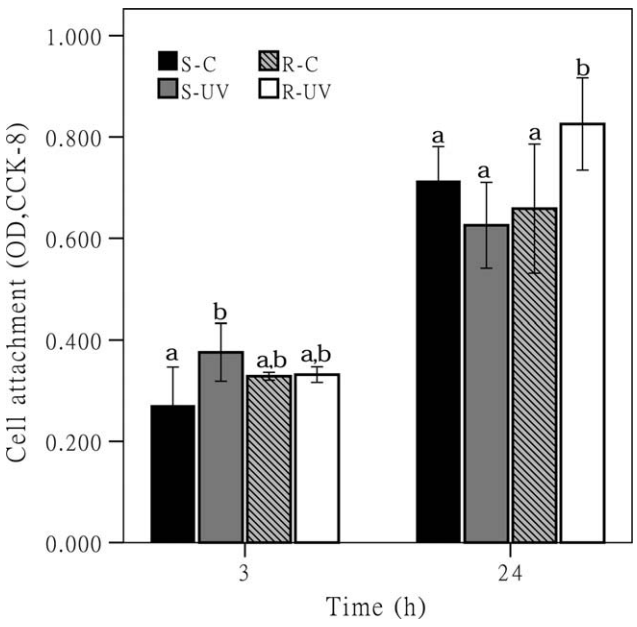

FIGURE 9. The quantity of HGFs on four disks measured by a CCK-8 assay. Cell attachment after cultured for 3 and 24 h. Experiments were repeated in triplicates, and each group has five disks. Data are shown as mean ± SD (n = 15). Identical letter shows no significant difference on each values ($p > 0.05$).

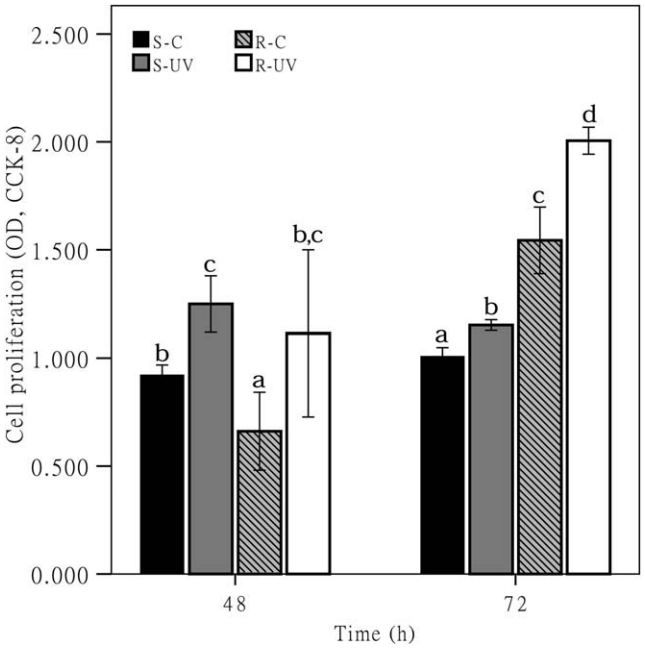

FIGURE 10. The quantity of HGFs on four disks measured by a CCK-8 assay. Cell proliferation after cultured for 48 and 72 h. Experiments were repeated in triplicates, and each group has five disks. Data are shown as mean ± SD (n = 15). Identical letter shows no significant difference on each values ($p > 0.05$).

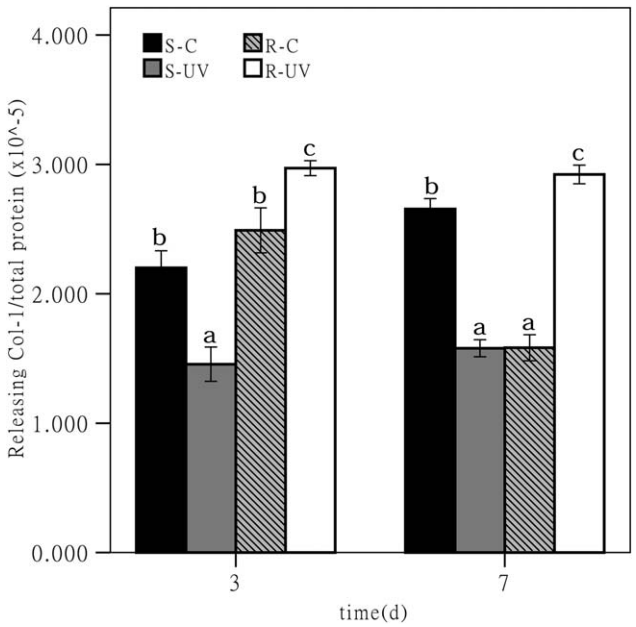

FIGURE 11. The amount of Col releasing into the culture medium of four disks, normalized on total protein, after cultured for 3 and 7 days. Experiments were repeated in triplicates, and each group has four disks. Data are shown as mean ± SD ($n = 12$). Identical letter shows no significant difference on each values ($p > 0.05$).

The present study only demonstrated the enhanced biological activity of HGFs, but the reaction of plaque formation, epithelial growth, and collagen arrangement is still unknown. The present results lead us to understand the complexity of soft tissue–implant interface. UV light treatment on zirconia abutment may provide guidance for further *in vivo* studies. These factors, both morphology and physicochemical properties, which may affect the biocompatibility of zirconia, are still the target of exploration.

CONCLUSION

The present study demonstrated that UV light-treated zirconia affected the behavior of HGFs, including cell adhesion, proliferation, and collagen release. This influence varied with surface roughness. After UV light irradiation, the rough surface promoted the fast 24 h adhesion and growth of HGFs after 48 and 72 h, as well as the enhanced cell morphology and the most collagen release. The smooth zirconia after UV light treatment enhanced only the 3 h adhesion, cell morphology, and proliferation. Surface morphology and hydrophilicity, as two interacted factors, both are governed on HGFs behavior. This indicated that UV light treatment might be a potential technique used on abutment surface modification, to get better peri-implant soft tissue barrier and to provide stable gingiva contour around implant restorations in esthetic zone.

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