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Different behavior of human gingival fibroblasts on surface modified zirconia: A comparison between ultraviolet (UV) light and plasma

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This study was to evaluate whether UV light irradiation and He plasma treatment of zirconia disks enhances its biocompatibility with human gingival fibroblasts (HGFs), and to compare the difference of two methods. Zirconia disks were prepared and divided into three groups: UVC light treatment (Group UV), He plasma (Group P), and control group. The surface morphology, wettability were analyzed. The cultured HGFs' adhesive density, morphology, proliferation and collagen synthesis were measured. After UV light and plasma treatment, contact angles decreased. HGFs' adhesion and proliferation in Group P was the highest ($p<0.05$) at each time point. HGFs on Group P also released the highest level of Col-1 after 3 and 7 days. Our study demonstrated that plasma and UV light treatment on smooth zirconia improved the hydrophilic property of surface in different mechanism and He plasma had the better effect on cells adhesion, proliferation, and especially on collagen synthesis.

Keywords: Ultraviolet light, Plasma, Zirconia, Human gingival fibroblasts, Peri-implant soft tissue

INTRODUCTION

In recent years, dental implant has achieved a high degree of clinical success in partial and total edentulous patient. Several *in-vitro* and animal studies have demonstrated that soft tissue attachment to the trans-mucosal portion of implant or abutment serves as a biological barrier, maintains natural shape of gingiva and protects the underlying bone, conferring resistance to the alveolar bone against resorption[1,2]. So, the interface between implant material and peri-implant soft tissue is also a vital factor for implant success[3]. The formation and maintenance of soft tissue barrier mainly depends on the response of peri-implant soft tissue to implant materials[4-6]. This applies not only to the structure and surface topography, but also to the biological properties. Surface energy, topography, and surface treatment techniques are among the factors impacting cell's adhesion, distribution, and migration at the soft tissue-implant interface. Therefore, more and more interest has focused on the modification of implant or abutment surfaces, to improve soft tissue sealing around them.

Ceramic materials like yttria-stabilized zirconia (TZP) have been introduced as a popular material for implant abutments. Its tooth-like color offers great esthetic benefits. *In vitro* studies, smooth zirconia showed lower bacterial deposition[7] and greater biocompatibility compared with titanium[8]. Zirconia has been recommended as the most preferred material in esthetic zone[9]. To become an ideal abutment, it should allow for rapid fibroblast cell proliferation and attachment, but reduced biofilm and bacterial adherence. A certain threshold of roughness (Ra=0.2 μm) is necessary[10]. Surface modification techniques have been introduced to study and improve the biological response of tissue. Many studies have investigated the effect of surface characteristics such as surface topography, surface chemistry, and surface energy on cellular response[11-13]. Surface physicochemistry is a vital factor that affect cells response to material interfaces. Many researches have investigated the relationship between the hydrophilicity of a material surface and cell attachment[14,15], and demonstrated that improved physicochemical properties, which enhanced the wettability, cell adhesion and proliferation would eventually result in a better biomechanical interaction between peri-implant tissues and implant materials[16,17]. Zirconia is a photocatalytic material[18], so ultraviolet (UV) light and plasma treatment both have been proved to be effective means of modifying its wettability[19,20]. The increasing wettability of zirconia also enhanced the behavior of osteoblasts[19,21,22] and oral keratinocytes[23], which benefits its application on implant surface modification to get greater osseointegration and soft tissue seal. Thus, plasma and UV treatment of zirconia surface are expected to enhance the attachment of

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human gingival fibroblasts (HGFs). Furthermore, these two methods may have different influence on zirconia surface. Few studies compare the different effects and mechanism of plasma and UV light treatment of zirconia on fibroblasts' behavior.

For the present study, the objective was to investigate the influence of plasma and UV light treatment of zirconia on the adhesion, proliferation and collagen synthesis of HGFs, and compared the different mechanisms of these two methods.

MATERIALS AND METHODS

Zirconia samples preparation and modification

Zirconia disks (20 mm in diameter and 2mm thick; Zenostar, Wieland Dental, Pforzheim, Germany) were first obtained using a cutting machine. Specimens were ground with silicon carbide papers using a grinding machine (AutoMet 300, Buehler, Lake Bluff, IL, USA). All specimens were ultrasonically cleaned for 15 min, with absolute ethanol and distilled water. After that, zirconia disks were divided into three groups:

Group UV; Zirconia disks 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 (λ=250±20 nm).

Group P; Zirconia disks were treated using an atmospheric room temperature plasma (ARTP) films treated instrument for 60 s (Tsinghua university, Beijin, China), with a voltage of 4.64–4.72 kV, frequency of 26 kHZ, and helium gas with a pressure of 4 L/min was used.

Group C; The rest of zirconia disks were stored at dark and dry condition as control.

Prior to the cell culture; All specimens were disinfected for 30 min with 75% alcohol.

Surface analysis

1. Measurement of surface roughness and morphology

Topographic analysis was performed by a Laser 3D profile measurement microscopy (VK-X200 series, KEYENCE, Osaka, Japan). The arithmetical mean surface roughness (Ra in μm) of each sample was measured five times at different areas. The average of five disks was used for the roughness of each group. Then zirconia disks were examined by scanning electron microscopy (SEM; S-4800, HITACHI, Tokyo, Japan). The object lens used was of 50× magnification.

2. X-ray diffraction (XRD) analysis

To determine crystalline structure, the disks were examined by XRD (D8 advance, Bruker AXS, Karlsruhe, Germany).

3. Measurement of surface wettability

Zirconia disks were examined by the contact angle of 1 μL H_2O droplet using a contact angle meter (SL200, Kino Industry, Boston, MA, USA). Three disks from each group were picked, and the average of three disks was used for the wettability of each group.

4. X-ray photoelectron spectroscopy (XPS) analysis

Electron spectroscopy for chemical analysis (ESCA) was used to evaluate the composition of the outermost surface. It was performed by XPS (ESCALAB 250, Thermo Fisher Scientific, DE, USA), 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 biopsies obtained from periodontal surgery of a periodontally healthy human subject with the advanced approval from Institutional Review Board of Peking University School of Stomatology (approval number: PKUSSIRB-2012060). Details of the cell culture protocols were described in our previous studies[24].

Measurement of cell density

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, Kumamoto, Japan). After 48 and 72 h, the proliferation of cells was measured. At each time point, the cells were washed with PBS for three times, and CCK-8 solution (100 μL CCK-8 per milli-liter cell culture medium) was added. After incubation at 37°C for 2 h, optical density (OD) of the solution was measured on a spectrophotometer (ELX808, BioTek, Winooski, VT, USA), with wavelengths at 450 nm. Experiments of all three groups were repeated in triplicate, and each group has 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 specimens were fixed in 95% alcohol for 30 min.

1. SEM analysis

Specimens were dehydrated in ascending ethanol series (ranging from 30 to 100% ethanol, three times each for 10 min at 4°C). Finally, the samples were sputter coated with gold palladium for 60 s at 60 mA (SCD050, Balzers, Liechtenstein).

2. Indirect immunofluorescence (IIF) analysis

Specimens were stained using FITC-phalloidin (actin filament green color, Sigma, St. Louis, MO, USA) and DAPI (nuclei blue color; Roche, Basler, Swiss). Fluorescence images were photographed using a confocal laser scanning microscopy (LSM710, Zeiss, Oberkochen, Germany). Five random images were obtained of each group of disks, and experiments of all three groups were repeated in triplicate. About 25 single cells were used to assess cell morphology. Cell area and perimeter were

quantified using an image analyzer (ImageJ, version 2, NIH, Bethesda, MD, USA).

Collagen release of HGF cells

The amount of type I collagen released into the cell culture medium was determined with a human collagen type 1 (Col-1) enzyme-linked immunosorbent assay kit (Bluegene Biotech, Shanghai, China). After 3 and 7 days of culture, 50 μL of cell culture medium were 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 OD was measured at 450 nm on a spectrophotometer (ELX808, BioTek). Total protein of cells on each disk were also measured using BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). The final collagen concentration was normalized on total protein. Experiments of all groups were repeated in triplicate, and each group has five 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, Armonk, NY, USA).

RESULTS

Surface analysis

With SEM observation, little difference in morphology could be found among all three groups. They all showed a relatively smooth morphology with some typical traces from grinding process (Fig. 1). The average roughness (Ra) of zirconia disks was 0.05±0.01 μm, and no significant difference was found after UV light and plasma treatment. XRD analysis showed that these disks fit the properties of zirconium Yttrium Oxide (Fig. 2), and even after UV and plasma treatment, the structure

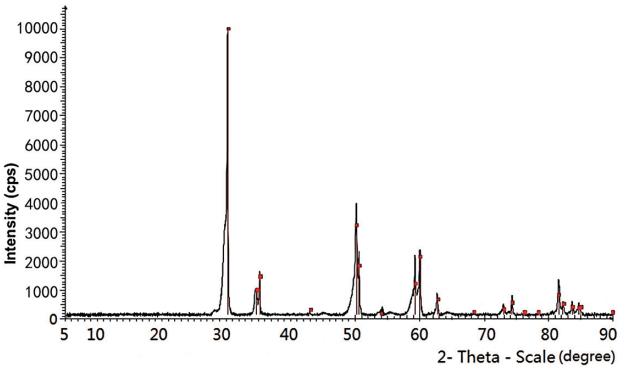

Fig. 2 XRD spectrum of zirconia disks. It is accord with zirconium yttrium oxide.

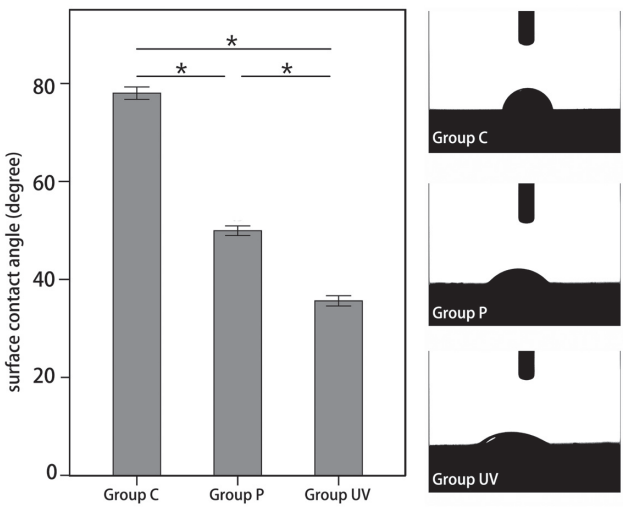

Fig. 3 After plasma and UV light treatment, the surface contact angles significantly decreased.
Data are shown as mean±SD (n=15) *p<0.05.

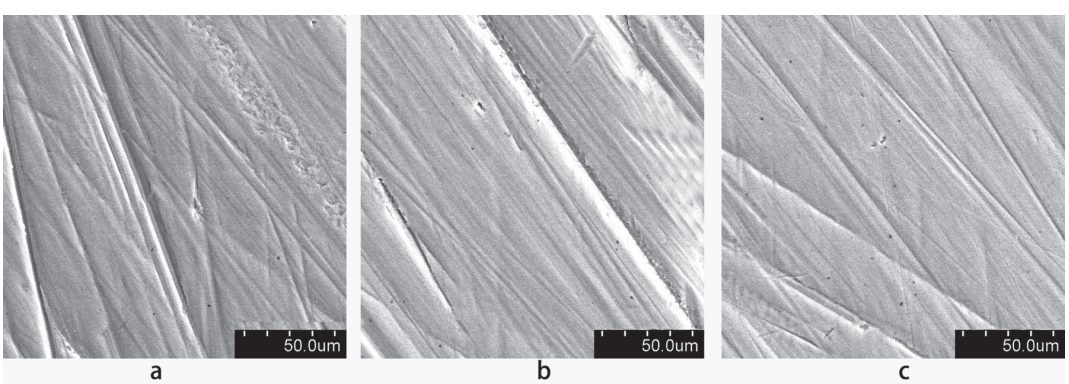

Fig. 1 Surface morphology of three groups of zirconia as SEM images.
(a) Group C, (b) Group P, (c) Group UV.

and tetragonal phase of ZrO_2 remained the same.

The contact angle of zirconia in Group C was about 78.03°. It decreased significantly after plasma and UV light treatment to 49.94° and 35.62°, respectively (Fig 3), which displayed more hydrophilic surfaces than control. UV-treated disks had even smaller contact angle compared with plasma.

The XPS analysis of zirconia specimens showed peaks of C1s, O1s, Ca2p3, N1s, Y3d and Zr3d (Fig. 4). The atomic percentage of C1s (at%) and O1s (at%) on the outermost surface of each group were listed in Table 1. C1s (at%) on Group UV decreased the most, so was the surface C/O ratio. The O1s spectra of the oxidized Zr metal was fitted with three symmetrical, mixed Gaussian-Lorentzian peak components: Dissolved (BE=530.1±0.1 eV), Oxide (BE=531.2±0.1 eV), and Hydroxide (BE=532.5±0.2 eV)[19] (Fig. 5). The relative area ratios of the three deconvoluted peaks are calculated and listed in Table 2. Group P also had the highest hydroxide ratio.

HGFs' adhesion, proliferation and collagen releasing

After 3 h of culture, the morphology of fibroblasts cultured on disks was shown on Fig. 6. Fibroblasts on control group had little cellular processes, while cells on treated surfaces were significantly larger and had more protrusions and intercellular interaction. Cells on Group P had the largest area, and the perimeters on treated groups were similar, both larger than control ($p<0.05$, Fig. 7).

The OD of attached HGFs on three groups was shown in Fig. 8. At every time point, HGFs on Group P had the highest OD ($p<0.05$). Cells on Group UV also had much higher OD than control after 72 h of culture ($p<0.05$). It showed an enhanced ability of cell proliferation on treated zirconia compared with control, and plasma treatment had even better effect.

The collagen released into the cell culture medium was measured after 3 and 7 days (Fig. 9). Our study normalized the concentrations of collagen on total

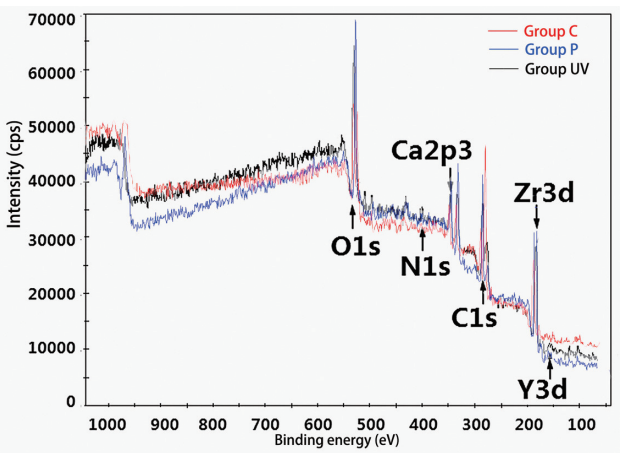

Fig. 4 XPS spectrum of zirconia in three groups.

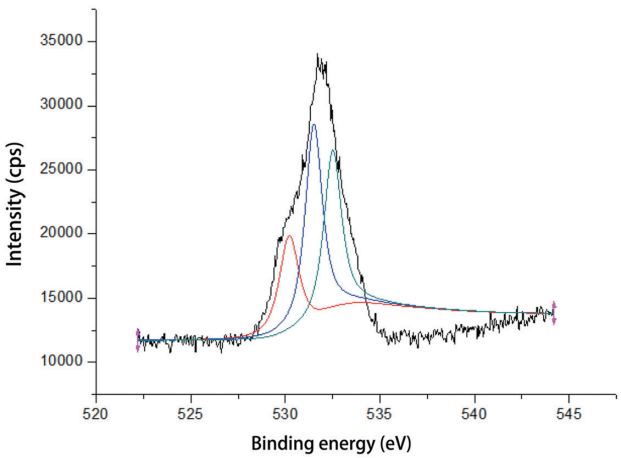

Fig. 5 The XPS O1s spectra of zirconia was fitted with three components.
Dissolved (BE=530.1±0.1 eV), Oxide (BE=531.2±0.1 eV), and Hydroxide (BE=532.5±0.2 eV).

Table 1 Atomic percentage of C1s and O1s on four surfaces

	C1s (at%)	O1s (at%)	C/O ratio
Group C surface	63.42	26.51	2.39
Group P surface	42.25	35.32	1.19
Group UV surface	43.03	32.32	1.33

Table 2 Area ratios of the deconvoluted peaks in O1s spectra

	Dissolved 530.1 eV	Oxide 531.2 eV	Hydroxide 532.5 eV
Group C surface	41.2	27.85	30.95
Group P surface	11.62	27.43	60.95
Group UV surface	25.79	31.94	42.27

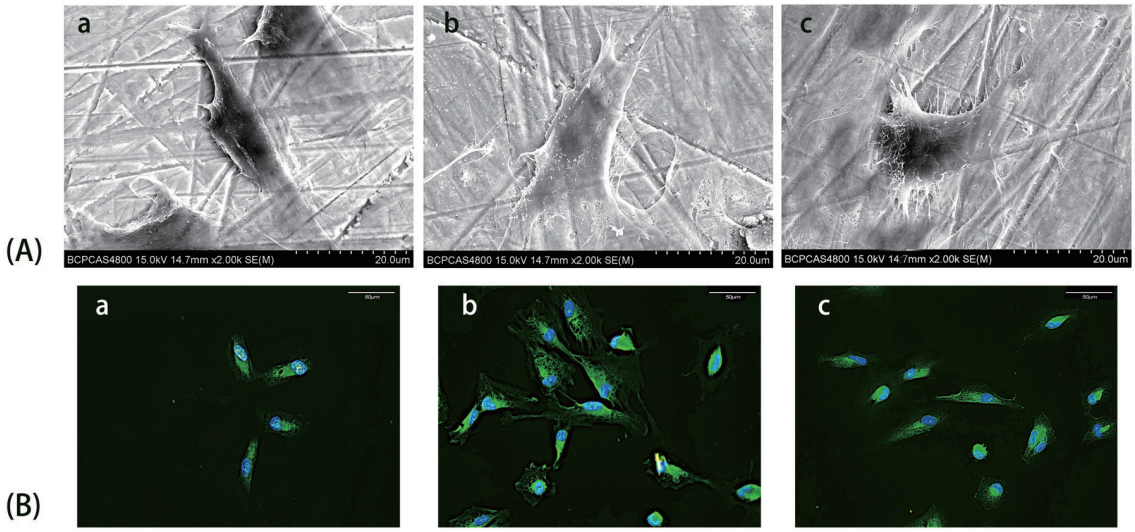

Fig. 6 Morphology of HGFs on three disks, after cultured for 3 h.
(a) Group C, (b) Group P, (c) Group UV, (A) SEM images, (B) IIF images, the HGFs were stained by FITC-phalloidin (green) and DAPI.

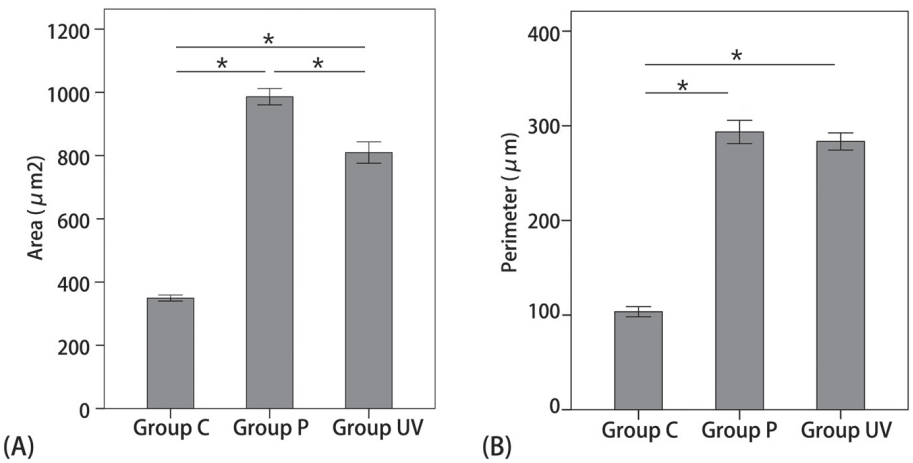

Fig. 7 Cell morphometric measurements.
The areas (A) and perimeters (B) of cells on three surfaces. Data are shown as mean±SD (n=25) *p<0.05.

cellular protein on each sample, which eliminated the effects of different cellular quantities. So, Col-1 in 7 days would be equal with or even lower than 3 days, because it showed how much Col-1 released by every single cell. The level of Col-1 in Group P was the highest, while Group UV had no difference with control (p>0.05). As a result, plasma treatment on zirconia advanced the ability of HGFs to release Col-1. While UV light irradiation did not improve cells' collagen producing.

DISCUSSION

Zirconia has become a promising abutment material as a substitute for titanium alloy. Its outstanding compatibility with peri-implant soft tissue concerning the surface chemistry and physical properties promote the favorable outcome. To obtain better soft tissues-zirconia integration, modification of surface is becoming more and more popular. Plasma and UV light treatment has been proven to enhance the behavior of soft-tissue cells by changing the hydrophilic property of zirconia-based implant materials[25]. Fibroblasts are the dominating cells in connective tissue and produce fibers and matrix. It was found that the peri-implant mucosa contained a large volume of collagen, and the inner zone which immediately lateral to the implant surface contained a higher amount of fibroblasts compared to the outer zone[26]. During the healing process after implant surgery, when HGFs once contacted a biomaterial surface, their adhesion proliferation and producing collagen would

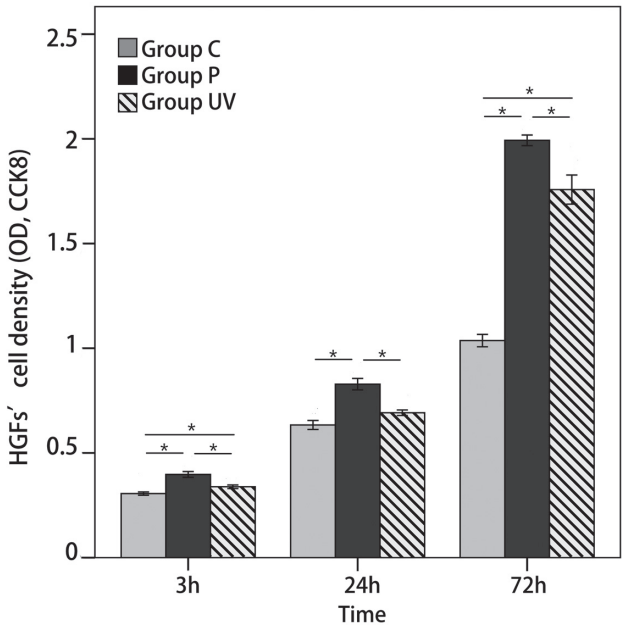

Fig. 8 The quantity of HGFs on three surfaces measured by CCK-8 assay, after cultured for 3, 24 and 72 h. Data are shown as mean±SD (n=15) *p<0.05.

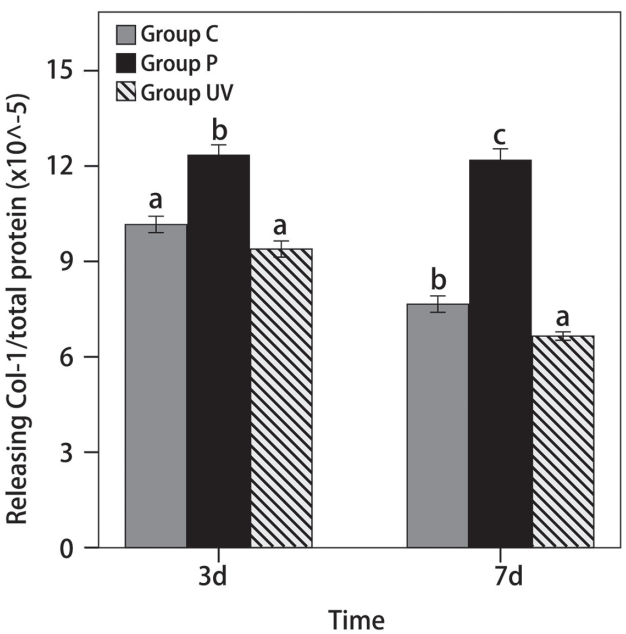

Fig. 9 The amount of col releasing into culture medium of three surfaces, normalized on total protein, after cultured for 3 and 7 days.
Data are shown as mean±SD (n=15) *p<0.05.

all affect the soft tissue sealing around abutment or the neck of implant. In this study, we focused on these major steps, and tried to further explore on which step UV light and plasma treatment really worked.

After 24 h UV light treatment and 60 s plasma, XRD analysis indicated that treatments did not affect the crystalline structure of TZP, suggesting that it is possible to modify zirconia surface without sacrificing the mechanical strength. The change of wettability in plasma group was not as much as UV group. The XPS analysis showed that after UV light treatment the decrease of C1s peak and the reduction of C/O were both more conspicuous than plasma group. It has been proved that removal of carbon content in the material surface was attributed to direct decomposition of carbon by UV treatment and photocatalytic activity of zirconia[17]. Due to this mechanism, UV light treatment may greatly increase the surface wettability, resulting in superhydrophilicity[20]. But in our study, the decrease of surface contact angle was not as much as previous study. It maybe because the surfaces in our study were much smoother than other studies[19,20,23]. For rough surface, the UV effects were more remarkable, which was in accordance with reports before. It may be due to the larger surface area of rough surface for absorbing UV light. In the other hand, after plasma treatment, the specimens had the highest peak of hydroxide (bonding energy of 532.5 eV), and the appearance of hydroxide would explain the increase of wettability in plasma group.

The remarkable change of hydrophilicity has significant influence on HGFs as well, cells' adhesion and proliferation improved after UV and plasma treatment. Spindle-shaped cells with pseudopod-like processes were more frequently seen in both treatment groups. Col-1 is cellular matrix produced by HGFs, and is the main composition of collagen in soft tissues. The ability of releasing Col-1 was also improved in plasma group. All this leads up to plasma-treated zirconia becoming the most favorable surface for HGFs' adhesion, proliferation and releasing collagen. Even though Group UV had better surface wettability, HGF's behavior was enhanced most after plasma treatment. It may be assigned to different mechanism of UV light and plasma. As the result of XPS in Fig. 4, the peak of C1s decreased the most in Group UV, while the peak of O1s, especially the peak of hydroxide, increased the most in Group P. The roles of basic hydroxyl OH (b) group on the osteoblast-titanium interactions have been proved[27]. Hydroxyl (–OH) and oxygen ($–O_2$) groups have been proved to form on the outermost layer when the hydrophilic oxide surface binds to water[28]. In the tissue fluid, the formation of hydroxylated oxide surface would improve the surface reactivity with the surrounding ions, amino acids, and proteins[29]. So, it will be logical to assume that even though the decrease of C1s peak and the hydroxide produced by surface treatment both increased zirconia's hydrophilicity, the hydroxide might have more effect on enhancing the biological behaviors of the HGFs.

Many studies have reported that high surface wettability would promote greater cell attachment. Superhydrophilic Ti and TZP disks enhanced osteoblasts' attachment, proliferation and differentiation[30]. But as seen in current study, it seems difficult to make a direct link between surface wettability and biocompatibility.

The relationship between surface wettability gradient and effect on cells' behavior has also been clarified by Lee *et al.*[31] that maximum adhesion and growth of fibroblast appeared with moderate hydrophilicity of the wettability gradient surface than onto the more hydrophobic or hydrophilic positions. Compared with surface wettability, surface biochemical components may be more important in improving cells' behavior. Previous study also demonstrated that hydroxyl groups created by plasma treatment enhanced adhesion and proliferation of human oral keratinocytes. Although UV treated specimens experienced carbon reduction and also obtained hydrophilicity like plasma treated specimens, this indicated that other factors including the surface energy and surface hydroxyl groups were involved in the phenomenon observed[23,25].

As we showed in Fig. 9, type I collagen in Group UV was approaching to Group C in 3 days, and even lower than control in 7 days. In previous study, we also found that UV light treatment on smooth zirconia did not improve collagen releasing in 7 days culture[24]. It may be related to time-variation of surface wettability after treatment. When zirconia samples were stored in different situation after UV light treatment, the contact angle of zirconia surface would gradually increase[20]. In our study, the relatively smooth zirconia surface we used was even more difficult to change its wettability than rough surfaces, so it maybe more difficult to maintain its hydrophilic property. Hence, the effects of UV light on HGF might be limited in short term. In addition, the mechanism of how the treated surfaces affect HGFs' collagen releasing was still unknown. We might only assume that the changes of hydrophilic property and element composition of zirconia surface affect the Col-1 releasing of HGFs in Group UV.

Above all, it may illustrate that surface wettability and chemical composition were both 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. The complicated link between surface properties and cells attachment still needs further researches to illustrate.

CONCLUSION

With the limitation of this study, it may be concluded that UV light and plasma treatment both enhanced zirconia's wettability and had influence on the behavior of HGFs with different mechanism. In contrast, 60 s He plasma had a better influence on HGFs's adhesion and proliferation, and especially on collagen synthesis. It indicated that better attachment of HGFs on plasma-treated surfaces, creating a more efficient soft tissue seal around dental implants.

CONFLICT OF INTEREST

All authors have no conflicts of interest.

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