Ultraviolet irradiation enhanced bioactivity and biological response of mesenchymal stem cells on micro-arc oxidized titanium surfaces

Zhipeng LI1,2, Juan YI1,2, Baoxin HUANG1,2, Xiayl WU1,2, Wei QIAO1,2, Xin LUO1,2 and Zhuofan CHEN1,2

1 Guanghua School of Stomatology, Hospital of Stomatology, Sun Yat-sen University, 56 Ling Yuan Road West, Guangzhou 510055, Guangdong, China
2 Guangdong Provincial Key Laboratory of Stomatology, 74 Zhong Shan Er Road, Guangzhou 510055, Guangdong, China
Corresponding author, Zhuofan CHEN; E-mail: dentistczf@163.com

This present study investigated the effect of ultraviolet (UV) irradiation on bioactivity of micro-arc oxidized (MAO) titanium surface in vitro by cell culture medium immersion test and interactions with rat-derived mesenchymal stem cells (MSCs). UV-irradiated MAO surface exhibited no obvious changes in surface roughness, morphology, and phase composition when compared with MAO-only surface. However, in cell culture medium immersion test, markedly more bone-like apatite was formed on UV-modified samples than on MAO sample. Rat bone marrow- and adipose tissue-derived MSCs cultured on UV-modified samples displayed accelerated attachment, significant higher levels of alkaline phosphatase (ALP) activity, and up-regulated osteogenesis-related mRNA expression than MAO sample. XPS results provided direct evidence that the amount of basic hydroxyl groups increased with UV irradiation time, which could be one of the key mechanisms underlying their improved bioactivity.

Keywords: Ultraviolet, Micro-arc oxidation, Hydrophilicity, Bioactivity, Mesenchymal stem cells

INTRODUCTION

Much research and considerable effort has been devoted to modifying the surface topography and chemical composition of titanium implants1) to improve their osteoconductivity and osseointegration. Micro-arc oxidation (MAO) is a relatively convenient and commonly used surface modification technique for clinically used titanium implants. Newly formed TiO2 coating, obtained via MAO, was both porous and firmly adherent to the titanium substrate, which is beneficial for the biological performance of titanium implants2). In addition, calcium (Ca) and phosphate (P) ions can be incorporated into the surface coating by controlling the composition and concentration of the electrolyte during the MAO process2-7). It has been reported that Ca- and P-containing surfaces enhanced both cell response in vitro6) and osseointegration in vivo6).

Recently, ultraviolet (UV) irradiation has been reported to improve the hydrophilicity of various modified titanium surfaces8-15), to the effect of improving the bioactivity7,8,10) and cell response11-14) of implants both in vitro and in vivo9,15). However, the mechanisms that underlie both hydrophilicity and biological response changes were still not fully understood and need further investigation.

Bioactive materials, defined as materials which form direct chemical bonds with bone when implanted into the living body, could stimulate bone formation and accelerate osseointegration16-18). Bioactivity of these materials is characterized by their ability to form bone-like apatite when implanted in a living body. Apatite formation in vitro can be estimated in vitro by immersion in simulated body fluid (SBF), a standard method widely used to test the bioactivity of bone-binding materials16-18).

The composition of SBF is different from that of human blood plasma in terms of blood proteins and biological macromolecules, and which has been proved to have significant effects on apatite formation19,20). On the other hand, calcium concentration in SBF is equivalent to that of total calcium in plasma. Such concentration level, however, is much higher than that of free calcium, which plays a very active role in apatite formation. The cell culture medium, which contains both macromolecules and calcium equivalent to free calcium in serum, could be considered as a better content source for bone-binding material’s bioactivity immersion test20). However, information is scarce on testing the bioactivity of modified titanium surfaces in cell culture medium.

The bioactivity of titanium implants is a prerequisite to achieving osseointegration, where mesenchymal stem cells (MSCs) are induced to proliferate and differentiate into osteoblasts, and produce direct bone apposition onto the implant surface. Therefore, MSCs are the preferred cellular source for testing cell and material interactions11,22,23). Bone marrow-derived mesenchymal stem cells (BMSCs) are commonly used in bone tissue engineering and testing of titanium materials. Recently, adipose-derived mesenchymal stem cells (AMSCs) were suggested to be a more convenient source of mesenchymal cells than BMSCs in terms of their abundance and accessibility. Although AMSCs were commonly thought to have less osteogenic potential than BMSCs24), it has been reported that when induced to osteogenic differentiation25), AMSCs could produce a calcified...
extracellular matrix\textsuperscript{25,26}—hence exhibiting comparable osteogenic potential as BMSCs. Unfortunately, little data is available on testing the osteogenic potential of AMSCs on modified titanium surfaces.

The purpose of the present study is to compare the changes in surface characteristics and bioactivity of MAO surface before and after UV irradiation. Bioactivity evaluations were carried out using cell culture medium immersion test and \textit{in vitro} biological response of rat bone marrow- and adipose tissue-derived MSCs.

**MATERIALS AND METHODS**

\textit{Preparation and surface modification of titanium samples}

Commercially pure Grade 2 titanium plates (INT, Baoji, China), of 10×10×1 mm dimensions, were used as MAO substrates. For pretreatment, all titanium plates were polished with successive grits of silicon carbide sandpapers of 280-, 360-, 400-, 600-, 800-, and 1000-grit. After ultrasonic cleaning with acetone, alcohol, and grit. After ultrasonic cleaning with acetone, alcohol, and deionized water, all pretreated titanium plates were dried at room temperature.

Titanium plates were divided into three groups, labeled as MAO, UV-2h, and UV-6h. MAO served as the control group, while UV-2h and UV-6h were experimental groups. For all the three groups, MAO was performed using a regulated DC power supply, which allowed automatic transition from constant current to constant voltage as described in a previous study\textsuperscript{27}). The electrolyte was prepared by dissolving 0.2 M calcium acetate monohydrate and 0.02 M β-glycerophosphate disodium salt pentahydrate in deionized water. Applied voltage, pulse frequency, duty cycle, and process duration were fixed at 400 V, 100 Hz, 30%, and 5 min respectively. An oxide layer of 20-μm mean thickness was formed on the titanium surface after micro-arc oxidation\textsuperscript{27}). After ultrasonic cleaning in alcohol and rinsing in deionized water, all MAO-treated titanium plates were dried by air in room temperature for 24 h. For the experimental groups, titanium plates were further UV-irradiated for 2 or 6 h, which was performed using a 1000 W, high-pressure UV lamp (Prosper, Taiwan, China) with a maximum intensity at 365 nm.

\textit{Surface characterization}

Surface morphology and elemental composition were examined by scanning electron microscopy (SEM; XL 30 FEG, Philips, The Netherlands) with energy dispersive X-ray (EDX) microanalysis. Elemental and chemical compositions of surface coatings were examined by X-ray photoelectron spectroscopy (XPS; Axis Ultra, Kratos Analytical, Manchester, UK). Phase composition was examined by X-ray diffraction (XRD; D/max-2400, Rigaku, Tokyo, Japan). Functional groups were identified using Fourier transform infrared spectroscopy (FTIR; Nicolet 6700, Thermo Fisher Scientific, USA). Contact angles were measured using the sessile drop method with Dataphysics Contact Angle System (DC-20, Dataphysics, Germany).

**Cell culture medium immersion test**

MAO, UV-2h, and UV-6h samples were stored in six-well cell culture plates and immersed in 10 mL of Dulbecco’s Modified Eagle’s Medium/Ham’s Nutrient Mixture F-12 (DMEM/F12; Gibco/Invitrogen, USA) cell culture medium supplemented with 10% fetal bovine serum (FBS; Gibco/Invitrogen, USA). Samples were incubated at 37°C under an atmosphere of 5% CO\textsubscript{2} and 95% air. The medium was refreshed every two days to keep the ion concentration stable. After 7, 14, and 21 days of immersion, all samples were dried at 70°C for 24 h. Apatite formation on titanium surfaces was examined using SEM and phase composition by XRD.

**Rat mesenchymal stem cell culture and flow cytometry**

Rat bone marrow-derived mesenchymal stem cells (BMSCs; OriCell\textsuperscript{TM}, Cyagen, USA) and adipose-derived mesenchymal stem cells (AMSCs; OriCell\textsuperscript{TM}, Cyagen, USA) were employed as seeding cells in the present study. Flow cytometric analysis of BMSCs and AMSCs at 3rd passage was performed using FACScan (Becton, Dickinson and Company, USA). Cells were harvested in 0.25% trypsin/EDTA and fixed for 30 min in 2% formaldehyde. After washing in a flow cytometry buffer, cells were incubated for 30 min in a flow cytometry buffer containing fluorescein isothiocyanate-conjugated monoclonal antibodies to the following CD antigens: CD34, CD45, CD44, and CD106. BMSCs and AMSCs were stained with a phycoerytrin-conjugated non-specific IgG to assess background fluorescence.

**Interactions between cells and material**

BMSCs and AMSCs at 3rd passage were used as seeding cells in the present study. MAO, UV-2h, and UV-6h samples were used for material studies, and all tests between cells and materials commenced immediately after UV irradiation. For cell attachment, morphology and proliferation assays, MSCs were cultured in the following growth medium: DMEM/F12 culture medium supplemented with 10% FBS, 100 IU/mL of penicillin, and 100 μg/mL of streptomycin.

For osteogenic differentiation assay, alkaline phosphatase (ALP) activity test and reverse transcription polymerase chain reaction (RT-PCR) were employed. MSCs were cultured in the following osteogenic medium: growth medium supplemented with 10 nM dexamethasone, 0.2 mM ascorbic acid, and 10 mM β-glycerophosphate.

1. **Cell attachment and morphology assay**

BMSCs and AMSCs were respectively seeded on MAO, UV-2h, and UV-6h samples in the growth medium at a cell density of 5×10\textsuperscript{4}/mL. After culturing for 30 min and 2 h, the samples were carefully rinsed with PBS three times and then fixed with 2% glutaraldehyde in PBS for 4 h. After dehydration with graded alcohols, samples were treated with tert-butyl alcohol and dried using a freeze dryer (UNICRYO MC, UniEquip, Germany). Cell adhesion and morphology on material surfaces were observed by SEM.
2. Cell proliferation assay
AMSCs and BMSCs were respectively seeded on MAO, UV-2h, and UV-6h samples in the growth medium at a cell density of 5×10^4/mL. After culturing for 1, 3, 5, and 7 days, cell viability was measured by MTT assay (ATCC, USA) as prescribed in manufacturer’s manual for measuring cell proliferation on disk specimens. Briefly, 100 uL of MTT (5 mg/mL) was added to each well and incubated at 37°C for 4 h. Then, MTT-containing medium was removed and 0.5 mL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals. After removing the titanium plates from culture wells, optical density of the solution was measured at a wavelength of 570 nm with a spectrophotometer (Model 680 Microplate Reader, Bio-Rad, CA, USA).

3. Alkaline phosphatase activity assay
AMSCs and BMSCs were respectively seeded on MAO, UV-2h, and UV-6h samples in the osteogenic medium at a cell density of 5×10^4/mL. Alkaline phosphatase (ALP) activity assay was performed by testing the transformation of p-nitrophenyl phosphate (p-NPP) into p-nitrophenol (p-NP). ALP activity of the MSCs was measured at 3, 7, 14, and 21 days after being induced to osteogenic differentiation. At each time period, cells were digested using 0.5 mL of 0.25% trypsin for 15 min, and then 1.5 mL of growth medium was added to stop digestion. Suspensions of released cells were centrifuged at 300 g for 5 min, and cell pellets were lysed by 100 μL of 0.1% Triton X-100 solution (Sigma-Aldrich, Italy) for three freeze-thaw cycles. After another centrifugation at 300 g for 5 min, 20 μL of supernatant was transferred into each well of a 96-well plate and incubated with 80 μL of ALP reagent (Sigma-Aldrich, USA) at 37°C for 30 min.

ALP activity was determined by measuring the absorbance of p-NP, which could be measured at a wavelength of 405 nm using the spectrophotometer. A standard curve was generated by creating a series of standard solutions of p-NP as references for measurement. A portion of the supernatant was also used to assess total protein concentration, which was performed by Pierce Micro BCA Protein Assay (Thermo Scientific, USA). Finally, ALP activity was normalized to total protein content and expressed as μmol of p-NP per milligram of protein.

4. Osteogenic gene expression analysis
BMSCs and AMSCs were respectively seeded on MAO, UV-2h, and UV-6h samples in the osteogenic medium at a cell density of 5×10^4/mL. At 7 and 21 days after osteogenic induction, total RNA from cells was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer’s instruction. Quantitative real-time reverse transcription PCR (qRT-PCR) for ALP, osteocalcin (OC), runt-related transcription factor 2 (Runx2), osterix, and β-actin was performed using the Sequence Detection System (ABI PRISM 7000, Applied Biosystems, CA, USA). Primers and internally labeled oligonucleotides for each cDNA are shown in Table 1. For PCR amplification of cDNA, an initial amplification using gene-specific primers was done with a denaturation step at 95°C for 5 min, followed by 39 cycles of denaturation at 95°C for 10 s, then primer annealing at 58°C for 20 s, and finally primer extension at 72°C for 30 s. When the cycling steps were completed, a final extension at 72°C for 5 min was carried out before the reaction was stored at 4°C. Reaction products were quantified by using serial dilutions of each sequence of known concentration to generate a standard curve. All samples were run in triplicate, and target gene expression was normalized to the amount of β-actin.

Statistical analysis
Five different samples randomly selected from MAO, UV-2h, and UV-6h groups were subjected to surface characterization by SEM and XPS as well as contact angle measurement. At each time point, three different samples randomly selected from MAO, UV-2h, and UV-6h groups were used for cell culture medium immersion test and cell attachment observation by SEM, and a representative figure was chosen for each. Other

Table 1  Primer sequences for polymerase chain reaction

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALP</td>
<td>Forward primer: ACCTAGACACAAGGCACCTC</td>
</tr>
<tr>
<td></td>
<td>Reverse prime: TTCCGATTCAACTCATCG</td>
</tr>
<tr>
<td>Osteocalcin</td>
<td>Forward primer: TCTCTGCTCACTCTGTCG</td>
</tr>
<tr>
<td></td>
<td>Reverse primer: GGAGTCTATTCACCACCTTAC</td>
</tr>
<tr>
<td>RUNX2</td>
<td>Forward primer: TCCCCGGGAACCAAGAAG</td>
</tr>
<tr>
<td></td>
<td>Reverse primer: CGGTCAGAGAACAAACTAGGTTTAGA</td>
</tr>
<tr>
<td>Osterix</td>
<td>Forward primer: CAATGACTACCCACCCCTTTCC</td>
</tr>
<tr>
<td></td>
<td>Reverse primer: ATGGATGCCCGCCTTGTA</td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward primer: TTCTCCGGGTTCCTGAAATGTTG</td>
</tr>
<tr>
<td></td>
<td>Reverse primer: CACTCTGAAGCCTTCCAGG</td>
</tr>
</tbody>
</table>
experiments were performed in triplicate and data were expressed as mean±SD. Statistical analysis was carried out using SPSS 15.0 software. For MTT and ALP activity assays, data were first checked for normal distribution and homogeneity of variance. They were then analyzed by one-way analysis of variance (ANOVA), and Bonferroni tests were used for comparison among groups at each time point. \( p<0.05 \) was considered to be statistically significant.

RESULTS

Surface characteristics

Figures 1 and 2 show the surface morphologies and XRD patterns of MAO samples before and after UV irradiation for 2 and 6 h, respectively. MAO surface (i.e., the control) appeared to porous with well separated and homogeneously distributed pores, the average size of which ranged between 1 and 2 μm (Fig. 1(a)). After UV irradiation for 2 and 6 h, there were no significant changes in surface morphology when compared with the control (Figs. 1(b) and (c)).

In Fig. 2, the Ti peaks of all samples were contributed from the titanium substrate. MAO surface was composed of anatase (A) as a predominant phase and a small amount of rutile (R). After UV irradiation for 2 and 6 h, there were no significant phase changes when compared with the control.

Figure 3 shows the FTIR spectra of MAO before and after UV irradiation at 400–4,000 cm\(^{-1}\) and 3,000–4,000 cm\(^{-1}\). MAO exhibited similar absorption spectra in the range of 3,700–4,000 cm\(^{-1}\) before and after UV irradiation. However, stronger absorption spectra at 3000–3700 cm\(^{-1}\) were observed for UV-2h and UV-6h samples than for the control. These absorption spectra were attributed to be partially due to water molecular adsorbed on the surface and partially due to hydroxyl groups bound to titanium surface, indicating that abundant hydroxyl groups were generated on MAO surface after UV irradiation\(^{28}\).

Table 2 shows mean roughness Ra, Rq, and Rz of MAO, UV-2h, and UV-6h samples. There were no statistically significant differences among the three samples \( (p>0.05) \), suggesting that no roughness change occurred during the UV irradiation procedure.

Deionized water droplet of 1 μm remained unspread on MAO sample, and contact angle was 78.3°±4.7°. After UV irradiation for 2 and 6 h, the water droplet spread and contact angle decreased to 28.7°±2.3° and 9.1°±1.1° respectively (Fig. 4). Decreases in contact angle indicated that MAO surface changed from being relatively hydrophobic to hydrophilic during the UV irradiation procedure.

Chemical composition

Table 3 shows the elemental compositions of MAO samples examined by XPS before and after UV irradiation. Detected elements consisted mainly of Ti, O, C, Ca, and P. A small amount of N was also detected. Figure 5 shows the XPS high-resolution spectra of O 1s, C 1s, Ti 2p, Ca 2p, and P 2p. In O 1s spectra, three Gaussian component peaks were fitted at 530.1, 531.3, and 532.8 eV (Figs. 5(a)–(c)). According to literature\(^{7,8}\), the first peak at 530.1 eV was assigned to O 1s in TiO\(_2\); second peak at 531.3 eV corresponded to O 1s in PO\(_4\)\(^{3-}\), C-O; and third peak at 532.8 eV was attributed to contribution from O 1s in C-O, O-C=O, basic hydroxyl groups. C 1s spectra were deconvoluted into four peaks at 284.8, 286.3, 287.9, and 288.9 eV (Figs. 5(d)–(f)). Predominant peak at 284.8 eV represented C 1s in C-C and C-H organics, and the other three peaks at 286.3, 287.9, and 288.9 eV were assigned to C 1s in C-O, C=O, and O-C=O respectively. In Ti 2p spectra, MAO sample

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**Fig. 1** SEM micrographs of rough MAO surfaces with well separated and homogeneously distributed pores before and after UV irradiation: (a) MAO; (b) UV-2h; and (c) UV-6h.

**Fig. 2** XRD patterns of MAO before and after UV irradiation: (a) MAO; (b) UV-2h; and (c) UV-6h. Ti: Titanium; A: Anatase phase; R: Rutile phase.
Fig. 3 FTIR spectra of MAO before and after UV irradiation at 400–4,000 cm\(^{-1}\) and 3,000–4,000 cm\(^{-1}\): (a) MAO; (b) UV-2h; and (c) UV-6h.

Table 2 Roughness of MAO before and after UV irradiation (mean±SD; n=3)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Ra</th>
<th>Rq</th>
<th>Rz</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAO</td>
<td>1.19±0.03</td>
<td>1.53±0.02</td>
<td>3.58±0.06</td>
</tr>
<tr>
<td>UV-2h</td>
<td>1.21±0.04</td>
<td>1.54±0.07</td>
<td>3.52±0.16</td>
</tr>
<tr>
<td>UV-6h</td>
<td>1.18±0.05</td>
<td>1.49±0.06</td>
<td>3.63±0.14</td>
</tr>
</tbody>
</table>

No statistically significant differences between three groups (ANOVA, \(p>0.05\)).

showed Ti doublet peaks at 458.7 eV (Ti 2p\(_{3/2}\)) and 464.4 eV (Ti 2p\(_{1/2}\)), which were well fitted to Ti 2p in TiO\(_2\). After UV irradiation for 2 and 6 h, more doublet peaks at 457.9 eV (Ti 2p\(_{3/2}\)) and 464 eV (Ti 2p\(_{1/2}\)) were identified, which corresponded to Ti 2p in Ti-OH (Figs. 5(g)–(i)).

Table 4 shows the percentage areas of deconvoluted peaks in O 1s and Ti 2p XPS spectra and percentage areas of deconvoluted C 1s peaks at 287.9 and 288.9 eV. Deconvoluted peak at 532.8 eV, attributed to contribution from O 1s in C=O, O-C=O, basic hydroxyl groups, became stronger after UV irradiation and increased with longer irradiation time. In contrast, percentage areas of deconvoluted C 1s peaks at 287.9 and 288.9 eV, assigned to C=O, O-C=O, decreased after UV irradiation. Therefore, deconvoluted 532.8 eV peak of O 1s became stronger because of an increase in basic hydroxyl groups.

Ca and P were present at approximately 10–15 at.%, and Ca/P ratio varied between 1.1 and 1.3. High-resolution spectra of Ca 2p peaks were deconvoluted into two peaks at 347.6 and 351.9 eV (Figs. 5(j) and (l)). High-resolution spectra of P 2p peaks were deconvoluted into two peaks at 133.4 and 134.4 eV, assigned to PO\(_3^–\) and HPO\(_4^{2–}\) respectively (Figs. 5(m) and (o)). Predominant
Table 3  Atomic concentrations and binding energies of elements in MAO, UV-2h and UV-6h samples by XPS

<table>
<thead>
<tr>
<th>Element</th>
<th>MAO At.% BE</th>
<th>UV-2h At.% BE</th>
<th>UV-6h At.% BE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ti</td>
<td>9.34 458.8</td>
<td>8.94 458.7</td>
<td>9.96 458.6</td>
</tr>
<tr>
<td>O</td>
<td>52.02 531.1</td>
<td>53.90 531.1</td>
<td>51.44 531.3</td>
</tr>
<tr>
<td>C</td>
<td>26.70 284.0</td>
<td>25.40 284.8</td>
<td>24.39 284.7</td>
</tr>
<tr>
<td>Ca</td>
<td>6.05 347.5</td>
<td>5.79 347.4</td>
<td>7.58 347.4</td>
</tr>
<tr>
<td>P</td>
<td>4.90 133.4</td>
<td>5.17 133.3</td>
<td>5.77 133.2</td>
</tr>
<tr>
<td>N</td>
<td>0.99 400.5</td>
<td>0.78 400.0</td>
<td>0.86 400.0</td>
</tr>
</tbody>
</table>

At. %, atomic concentration; BE, binding energy (eV).

Fig. 5  Deconvoluted XPS peaks of O 1s (a–c), C 1s (d–f), Ti 2p (g–i), Ca 2p (j–l), and P 2p (m–p) for MAO before and after UV irradiation: MAO (a,d,g,j,m), UV-2h (b,e,h,k,o), UV-6h (c,i,l,p).
Table 4 Percentage areas of deconvoluted peaks in O 1s and Ti 2p XPS spectra and percentage areas of deconvoluted C 1s peaks at 287.9 and 288.9 eV of MAO, UV-2h, and UV-6h samples

<table>
<thead>
<tr>
<th>Groups</th>
<th>Deconvoluted peaks in O 1s XPS spectra (%)</th>
<th>Deconvoluted peaks in Ti 2p XPS spectra (%)</th>
<th>Deconvoluted peaks in C 1s XPS spectra (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>530.1 531.3 532.8</td>
<td>458.7 457.9</td>
<td>287.9 288.9</td>
</tr>
<tr>
<td>MAO</td>
<td>40.6 48.4 11.0</td>
<td>100.0 0</td>
<td>6.5 4.4</td>
</tr>
<tr>
<td>UV-2h</td>
<td>33.2 52.7 14.1</td>
<td>96.5 3.5</td>
<td>4.8 4.3</td>
</tr>
<tr>
<td>UV-6h</td>
<td>29.6 46.1 24.3</td>
<td>91.9 8.1</td>
<td>3.2 4.3</td>
</tr>
</tbody>
</table>

Fig. 6 SEM images of samples immersed in cell culture medium.
(a) and (b) MAO immersion for 21 days; (c)–(e) UV-2h immersion for 7, 14 and 21 days and respectively in high magnification in (f)–(h); (i)–(k) UV-6h immersion for 7, 14, and 21 days and respectively in high magnification in (l)–(n).
peak at 133.4 eV of P 2p and 347.6 eV of Ca 2p corresponded to the peak positions expected of calcium phosphate compounds.

**Apatite formation ability in cell culture medium**
MAO surface remained porous without any crystal deposition after immersion in cell culture medium until 21 days, indicating that no bone-like apatite could be induced on MAO surfaces (Figs. 6(a) and (b)). On UV-2h sample, small crystals covered approximately 50% of surface area after medium immersion for 7 days. After longer immersion up to 14 and 21 days, the crystals grew larger and covered more surface area (Figs. 6(c)–(h)). Compared with UV-2h sample, UV-6h sample showed even greater crystal formation ability. After 7 days of immersion in the medium, small crystals were detected and covered approximately 70% of surface area. Crystal size and covered area also significantly increased with immersion time (Figs. 6(i)–(n)).

Figure 7 shows the XRD spectra of UV-6h sample immersed in cell culture medium at different time periods. New XRD peaks ascribed to apatite appeared in UV-6h sample after immersion for 14 days, indicating that the crystals seen in Fig. 6 were apatite crystals. Peak intensity relative to apatite increased with immersion time due to growth of apatite on the surface.

**Characterization of BMSCs and AMSCs**
Both BMSCs and AMSCs did not express hematopoietic lineage markers CD34, CD45, and CD106. However, both cells were positive for CD44, which was defined as an important marker for cells of mesenchymal origin.

**Table 5** Percentages of expressive cell markers of BMSCs and AMSCs by flow cytometry

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>CD34</th>
<th>CD44</th>
<th>CD45</th>
<th>CD106</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMSCs</td>
<td>0.5%</td>
<td>99.7%</td>
<td>0.2%</td>
<td>3.4%</td>
</tr>
<tr>
<td>AMSCs</td>
<td>0.2%</td>
<td>99.8%</td>
<td>0.1%</td>
<td>5.1%</td>
</tr>
</tbody>
</table>

Table 5 shows the expressive percentage of each marker for both cells.

**Interactions between cells and material**

1. **Cell attachment and morphology**
   Figure 8 shows the typical morphologies of both BMSCs and AMSCs attached to the surface at 0.5 h and 2 h. At 0.5 h, the cells just attached to the substrate after seeding; at 2 h, filopodia started to stretch out and adhere to the substrate. Cytoplasmic extensions and filopodia stretching out were significantly increased on UV-modified surfaces when compared with the control, indicating that UV modification expedited cell attachment and spreading.

2. **Cell proliferation**
   For both BMSCs and AMSCs, quantitative assessment of proliferation (MTT assay) showed similar proliferation rates on MAO, UV-2h, and UV-6h samples (ANOVA, \( p > 0.05 \)). These results indicated that UV irradiation exerted neither significant enhancement nor inhibition effect on MSCs' proliferation (Fig. 9).

3. **ALP activity**
   Figure 10 shows the ALP activities of BMSCs and AMSCs cultured on MAO, UV-2h, and UV-6h samples. For both cell lines and on all samples, ALP production increased with the culture time from 3 to 14 days, and then decreased from 14 to 21 days. In Fig. 10(a), ALP activity of BMSCs cultured on UV-2h and UV-6h was significantly higher than that on control at 7 days (\( p < 0.05 \)), and that on UV-6h also was also significantly higher than the control at 14 days (\( p < 0.05 \)). In Fig. 10(b), ALP activity of AMSCs cultured on UV-2h and UV-6h was significantly higher than that on control at both 7 and 14 days (\( p < 0.05 \)). However, for both BMSCs and AMSCs, there were no statistically significant differences in ALP activity cultured on MAO, UV-2h, and UV-6h at 3 and 21 days.

4. **Osteogenic gene expression**
   Gene expressions of Runx2, Osterix, ALP, and OC, which are involved in osteogenic differentiation, were analyzed for BMSCs and AMSCs and normalized to \( \beta \)-actin gene. In Fig. 11, time-dependent up-regulated expression levels of all target osteogenic genes from 7 days to 21 days were observed for both BMSCs and AMSCs. RT-PCR analysis showed that throughout the culture period, the expression levels of Runx2, Osterix, ALP, and OC in both BMSCs and AMSCs were up-regulated after UV irradiation when compared with...
Fig. 8 SEM micrographs for cell attachment. (a)–(f) Morphology of BMSCs attached to MAO, UV-2h, and UV-6h surfaces at 0.5 and 2 h respectively; (g)–(i) Morphologies of AMSCs attached to MAO, UV-2h, and UV-6h surfaces at 0.5 and 2 h respectively.

Fig. 9 MTT assay results for cell proliferation of: (a) BMSCs; and (b) AMSCs. Data are shown as mean±SD (n=3). There are no statistically significant differences between groups at each time point (p>0.05).
Fig. 10  ALP activity results of cells: (a) BMSCs; and (b) AMSCs. Data are shown as mean±SD (n=3), * indicates a statistically significant difference (p<0.05).

Fig. 11  Osteogenic gene expression levels in BMSCs and AMSCs as obtained by qRT-PCR: (a) Runx2; (b) Osterix; (c) ALP; and (d) OC. Data are shown as mean±SD (n=3).

the control, except the expression levels of Runx2 of AMSCs at 7 days and OC of BMSCs at 7 days in UV-2h group. Target gene expression levels were also higher in UV-6h group than in UV-2h group, except for ALP of BMSCs at 21 days, indicating that up-regulated osteogenic gene expression also correlated with UV irradiation time.
DISCUSSION
The present in vitro study revealed two major findings:
(1) UV irradiation improved the hydrophilicity of MAO surface and enhanced its apatite formation ability in cell culture medium; (2) UV irradiation accelerated early cell adhesion and osteogenic differentiation of both BMSCs and AMSCs on MAO surface.

A major target of implant surface modification is to accelerate and improve osseointegration. Sandblasted and acid-etched titanium surfaces reportedly accelerated and promoted osseointegration and increased bone-implant contact areas. Such improvements were reportedly brought about by an increase in hydrophilicity and a decrease in adsorbed hydrocarbons at the surface. Although the underlying mechanism remained to be fully elucidated, hydrophilicity was purportedly one of the crucial factors. UV irradiation has been proved to improve the hydrophilicity of titanium surfaces. Thus, it might also be a relatively convenient way to improve the bioactivity of titanium surfaces. In our present study, UV irradiation has been confirmed not only to generate hydrophilicity on MAO surface but also to improve its apatite formation ability in cell culture medium.

It has been reported in previous studies that UV irradiation improved the apatite formation ability of MAO surfaces in SBF. Apatite formation in vivo, however, is a much more complicated process than that which occurs in SBF. Therefore, cell culture medium supplemented with fetal bovine serum was employed as the immersion fluid in the present study. Compared with SBF, there were several advantages in using cell culture medium as an in vitro testing medium. First, it offers a calcium concentration which more closely mimics that in vivo. Secondly, organic biological molecules (such as proteins) which may influence initial apatite formation, are included in the medium.

Contrary to the results of previous studies which investigated apatite formation on MAO surfaces immersed in SBF, no apatite was formed on the MAO surface when immersed in cell culture medium in the present study. However, UV irradiation of the selfsame MAO surface led to significant apatite formation in the same cell culture medium. Basic hydroxyl groups generated during UV irradiation might be the reason for improved apatite formation. FTIR revealed strong absorption peaks at 3,000–3,700 cm⁻¹, which confirmed the existence of abundant hydroxyl groups on MAO surface after UV irradiation.

There are two kinds of hydroxyl groups on titanium surface — namely, negatively charged acidic hydroxyl groups and positively charged basic hydroxyl groups. XPS analysis revealed that UV-generated hydroxyl groups corresponded to O 1s at 532.8 eV, rendering them as positively charged basic hydroxyl groups. These basic hydroxyl groups resulted in a positively charged MAO surface, which caused negatively charged phosphate ions to be adsorbed on its surface. As the phosphate ions began to accumulate, the surface became negatively charged and combined with the positively charged calcium ions to form calcium phosphate. Calcium phosphate eventually transformed into stable crystalline apatite. This mechanism well explained the improved apatite formation ability on MAO surface after UV irradiation.

Interestingly, XPS revealed that not only the deconvoluted peak O 1s at 532.8 eV assigned to basic hydroxyl groups increased, but also two new deconvoluted peaks Ti 2p at 458.0 eV and Ti 2p at 464.0 eV assigned to Ti-OH. Percentage areas of these two new Ti peaks also increased with UV irradiation time. Therefore, UV-6h surface exhibited enhanced apatite formation ability when compared with UV-2h surface.

Osseointegration of an implant material depends on cell-material interactions, especially cell adhesion to material surface. Various cellular sources, such as MCT3T3 osteogenic cell lines, mature osteoblasts, and MSCs, have been employed to evaluate the biocompatibility of titanium materials in numerous studies. Osteoblasts exist only in the internal surface of cortical bone, and their quantity and proliferation ability are limited. Mesenchymal stem cells, on the other hand, play a more important role in osseointegration by continuously migrating, proliferating, and differentiating into osteoblasts. Therefore, MSCs have been suggested to be a good candidate for the evaluation of titanium materials' biocompatibility in cell attachment, proliferation, and differentiation studies.

In the present study, SEM was used to evaluate the attachment of MSCs to titanium surface. Compared with confocal laser scanning microscopy, which is commonly used to observe the cytoskeletal arrangement and expression of focal adhesion proteins, SEM also exhibited superiority in observing the filopodia and the direct connection between cells and material surface. Surface topography, roughness and other surface properties, such as surface charge and wettability, are generally recognized as crucial factors that influence cell-material interactions. Surface topography influences cell adhesion by offering focal adhesion points for attachment, and that increased surface roughness promotes cell adhesion, proliferation, and osteogenic differentiation. In the present study, MAO surface showed no significant changes in surface topography and roughness before and after UV irradiation. However, promotion of early cell adhesion was confirmed by SEM results in the present study.

Due to its bioinert nature, titanium does not directly interact with biological molecules and cells. Titanium surfaces require ionic bridges, especially divalent cations such as Ca²⁺, to attract proteins and cells. However in the present study, UV irradiation generated abundant positively charged basic hydroxyl groups, which formed a positively charged MAO surface. This surface thus
served as a direct chemoattractant to cells without divergent cations and directly attract negatively charged proteins and cells. Through which, early adhesion of cells on UV-irradiated surface was promoted.

AMSCs were reportedly inferior in differentiating into osteoblasts when compared with BMSCs—in terms of ALP activity and matrix mineralization into osteoblasts when compared with BMSCs—-in terms of ALP activity and matrix mineralization. However, AMSCs were also reported to be a good candidate for bone tissue engineering and had shown similar capability in differentiating into mature osteoblasts. In the present study, qRT-PCR analysis revealed a higher level of osteogenic commitment in BMSCs than in AMSCs, with respect to the expression levels of Runx2, Osterix, ALP, and OC on MAO, UV-2h, and UV-6h surfaces. Nonetheless, BMSCs and AMSCs cultured on UV-modified surfaces exhibited up-regulated expression of these osteogenic genes than the control at different time points, suggesting that UV-modified MAO surface promoted the osteogenic differentiation of both BMSCs and AMSCs. Further studies on variables such as cell origin, osteogenic induction cell culture medium, and induction time might help to provide more insight into the osteogenic potential of BMSCs and AMSCs.

Against the background of the above discussion and comparison with studies regarding the effect of UV irradiation on bioactivity of titanium surfaces, it was understandably difficult make definitive conclusions because of contradicting results arising from the following causes: (1) disparities in UV light intensity and irradiation duration; (2) variations in surface composition and topography of titanium substrate; (3) variations in bioactivity testing method. Therefore, further research on the effect of UV irradiation on improving bioactivity should focus on the effects of strength, wavelength, and duration of UV light treatment, as well as differences in the surface chemistry and topography of the titanium substrates used, which may also result in different biological responses.

CONCLUSIONS

In the absence of other surface characteristics’ changes, the hydrophilicity property of MAO surface was significantly improved after UV irradiation. Apatite was induced in both UV-2h and UV-6h samples in cell culture medium after 7 days, but not in MAO sample even after 21 days. XPS results provided direct evidence that the amount of basic hydroxyl groups increased with UV irradiation time, which could be one of the key mechanisms responsible for improved bioactivity. UV-irradiated MAO titanium surface also promoted MSCs’ initial attachment and osteogenic differentiation, suggesting that UV irradiation could be a trustworthy and relatively convenient method to improve the bioactivity of MAO-processed titanium surfaces.

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