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## H<sub>2</sub>O<sub>2</sub>/HCl and heat-treated Ti-6Al-4V stimulates pre-osteoblast proliferation and differentiation

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The purpose of the present study was to evaluate the bioactivity of chemical treatment of titanium alloy (Ti-6Al-4V) in vitro. Smooth-surface discs of Ti-6Al-4V were used in this study. Sandblasted, dual acid-etched and H<sub>2</sub>O<sub>2</sub>/HCl heat-treated discs were set as test group, and sandblasted, dual acid-etched discs as control group. SEM and XRD analysis revealed a porous anatase gel layer on rough surface in the test group and a rough surface in the control group. Mouse pre-osteoblasts (MC3T3-E1 cells) were cultured on these 2 group discs, and then cell proliferation and differentiation were examined 4 days, 7 days, and 14 days after cell seeding. Cell proliferation was greatly stimulated at all time points when cultured in test group ( $P < .05$ ). The alkaline phosphatase (ALP) activity and osteocalcin (OC) production were much higher in the test group compared with the control group at every time point investigated ( $P < .05$ ). Furthermore, in the test group, the expressions of alkaline phosphatase-2, osteocalcin, and collagen type I alpha 1 mRNAs were significantly up-regulated as compared with those in the control group ( $P < .05$  or  $P < .01$ ). The results suggested that H<sub>2</sub>O<sub>2</sub>/HCl and heat-treatment might facilitate better integration of Ti-6Al-4V implants with bone. (*Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2009;108:368-375)

Titanium (Ti) and its alloys are widely used in endosseous dental implants because of their excellent mechanical properties and superior biocompatibility. However, Ti and its alloys are generally considered to be bio-inert and not likely to form direct chemical bonding with bone. Surface coating and chemical treatment of these metals are 2 main approaches to enhance bone formation around dental implants. Despite many kinds of bioactive materials used to coat the surfaces of dental implants,<sup>1-5</sup> the one most extensively used is hydroxyapatite (HA) plasma-spray coating. But HA coating on Ti and its alloys by plasma-spray technique is usually degradable in body environments, with low fatigue strength and weak adherence to metallic substrates.<sup>6,7</sup>

Chemical treatment seems to be a simple and effective technique to achieve a bioactive titanium surface and therefore has been widely investigated in recent years.<sup>8-15</sup> NaOH and H<sub>2</sub>O<sub>2</sub> solutions are the most fre-

quently used reagents in the chemical treatment of dental implants. A sodium titanate gel layer was produced on a Ti surface when treated with NaOH solution, and that layer was bioactive, which could induce the deposition of bone-like apatite during soaking in simulated body fluid.<sup>11</sup> But the bioactivity of sodium titanate gel was heavily dependent on ion release from the gel, and its apatite deposition ability was completely lost after 1 day of prestaking in distilled water.<sup>11</sup> Concerning H<sub>2</sub>O<sub>2</sub> treatment, Wang et al.<sup>8,13,16</sup> extensively studied to yield a titania layer on Ti surface owing to the chemical treatment with H<sub>2</sub>O<sub>2</sub>/HCl solution and subsequent heating at a moderate temperature. The formed dioxide gel layer was able to induce rapid apatite deposition when soaked in simulated body fluid; moreover, its bioactivity depended on the gel's structure and was not degradable in aqueous solution.<sup>11</sup>

Roughened surfaces have been used to increase the total surface area for osseous apposition and thus to enhance bone formation around dental implants.<sup>17,18</sup> Ti alloys, such as Ti-6Al-4V, are being used as implants because of their superior mechanical properties to pure Ti. However, the widely studied substrates treated with H<sub>2</sub>O<sub>2</sub>/HCl solution and heating are almost the commercial pure Ti metals with smooth surfaces. To our best knowledge, the bioactivity of Ti-6Al-4V with rough surface treated with H<sub>2</sub>O<sub>2</sub>/HCl solution and heating still needs to be elucidated. In the present study, we aim to develop a bioactive surface on Ti alloy (Ti-6Al-4V) and investigate its in vitro bioactivity.

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## MATERIALS AND METHODS

### Surface modification of the Ti-6Al-4V discs

Flat discs,  $10 \times 10 \times 1$  mm and  $25 \times 25 \times 1$  mm in size, of Ti-6Al-4V (Xihu Biomaterial Research Institute, Hangzhou, China) were used in this study as substrate materials. All the substrates were first sandblasted with large grit (green silicon carbide) at 4 MPa pressure, then ultrasonically cleaned in acetone, 75% alcohol, and distilled water for 15 minutes each. After that, the substrates were chemically treated with a solution containing 0.11 mol/L HF and 0.09 mol/L HNO<sub>3</sub> at room temperature for 10 minutes, and dried in an oven at 50°C for 24 hours. Then, the substrates were treated with a mixed solution composed of 5.80 mol/L HCl and 8.96 mol/L H<sub>2</sub>SO<sub>4</sub> at 80°C for 30 min and dried in an oven at 50°C for 24 hours. After that treatment, half of the substrates were randomly selected and set as control group. The remaining discs were further treated with another solution containing 8.8 mol/L H<sub>2</sub>O<sub>2</sub> and 0.1 mol/L HCl at 80°C for 20 minutes, rinsed with distilled water, and dried in an oven at 50°C overnight, and finally heated in air at 400°C for 1 hour and allowed to cool in an electric furnace. And these discs were set as the test group.

Before cell culture, all the specimens including the control group and test group were ultrasonically cleaned in acetone, 100% alcohol, and distilled water for 15 minutes each, and sterilized with ultraviolet light for 1 hour.

### Surface analysis of the Ti-6Al-4V discs

The surfaces of the discs were characterized by field-emission scanning electron microscopy (FSEM FEI, SIRION100, Amsterdam, Holland). Crystal structure of the surfaces was analyzed by low-angle x-ray diffractometry. Low-angle x-ray diffractometry patterns were recorded with a Rigaku RAD-II diffractometer using CuK $\alpha$  radiation operating under 40 kV and 25 mA acceleration at an angle of incidence of 1°.

### Cell culture

Mouse pre-osteoblast cells (MC3T3-E1) were cultured in alpha-minimum essential medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco). The complete medium was replaced every 2 to 3 days and confluent cells were subcultured through trypsinization. To study the cell proliferation and differentiation of MC3T3-E1 on Ti-6Al-4V discs with different treatments, the cells were seeded at a density of  $1 \times 10^5$  or  $1 \times 10^4$  cells/well in 6- or 24-well cell culture plates (Corning, USA), respectively, to which the Ti-6Al-4V discs had been added. After 1 day of culture, the Ti-6Al-4V discs were transferred to new multiwell cell culture plates so that the cells adhering to cell culture

plates instead of Ti-6Al-4V discs could be minimized. The cells were then cultured for 2 weeks at 37°C in a humidified 5% CO<sub>2</sub>/95% air atmosphere with cell culture medium. The medium was replaced every 3 days, with sample analysis or preparation for qualitative assessment at days 4, 7, and 14. Each experiment was performed in triplicate ( $n = 3$ ) for each group and repeated 3 times; however, examiners were blind to the detailed groups.

### Cell proliferation

For the evaluation of cell proliferation, cells were cultured on Ti-6Al-4V discs ( $10 \times 10 \times 1$  mm) in 24-well cell culture plates for 4, 7, and 14 days. Cell proliferation on those specimens were determined by the measurement of total DNA content in cell layers with a PicoGreen dsDNA Quantitation Kit (Invitrogen, USA). At each time point, after removing medium, specimens were washed with PBS and covered by 300  $\mu$ L Na Citrate buffer solution containing 50 mM Na Citrate and 100 mM NaCl, and stored at -80°C until assay. After thawing at room temperature, the cells were scraped and sonicated. A 100- $\mu$ L volume of cell lysate was mixed with 100  $\mu$ L DNA binding fluorescent dye solution. After several minutes, a fluorescence spectrometer (Sepctra M2, Molecular, USA) was used to read the fluorescent intensity of the mixed solution at an excitation wavelength of 480 nm and emission wavelength of 520 nm against a standard curve.

### Alkaline phosphatase activity

The alkaline phosphatase (ALP) activity was measured with a commercial phosphatase substrate kit (Wako, Japan). Following gentle removal of culture medium and washing with PBS, the cells cultured on discs ( $25 \times 25 \times 1$  mm) in 6-well cell culture plates were lysed by incubation with ALP lysis buffer (CellLytic M, Sigma, USA). A 20- $\mu$ L volume of cell lysate mixed with 100  $\mu$ L working assay solution was shaken for 1 minute with a plate mixer, and then incubated at 37°C for 15 minutes. With the addition of 80  $\mu$ L stop solution to each well (96-well cell culture plate), reaction was terminated. After that, the mixtures were shaken for another 1 minute and the resulting optical densities were measured at 405 nm with a spectrophotometer. Total protein was determined with a BCA protein assay kit (Beyotime, China). ALP activity was expressed as nanomoles of p-nitrophenol liberated per microgram of total cellular protein per hour.

### Osteocalcin release

The production of osteocalcin by differentiated cells was measured as the release of extracellular matrix protein in the culture medium using Mouse Osteocalcin

**Table I.** Forward (F) and Reverse (R) primers for target genes

Gene name	Amplicon length (bp)	5'-3' primer sequence
AKP-2(NM007431)	164	F 5'-TGCCTACTTGTGTGGCGTGAA-3' R 5'-TCACCCGAGTGGTAGTCACAATG-3'
OC(NM007541)	178	F 5'-AGCAGCTTGGCCAGACCTA-3' R 5'-TAGCGCCGGAGTCTGTTCACTAC-3'
Colla1(NM007742)	153	F 5'-ATGCCGCGACCTCAAGATG-3' R 5'-TGAGGCACAGACGGCTGAGTA-3'
$\beta$ -actin(NM007393)	131	F 5'-TGACAGGATGCAGAAGGAGA-3' R 5'-GCTGGAAGGTGGACAGTGAG-3'

AKP-2, alkaline phosphatase-2; OC, osteocalcin; Colla1, collagen type I, alpha 1.

EIA Kit (Biomedical Technologies, USA). Briefly, a 25- $\mu$ L cell culture medium sample and 100  $\mu$ L osteocalcin antiserum were placed in a 96-well EIA plate and incubated at 4°C for 18 to 24 hours. The well was washed with wash buffer and 100  $\mu$ L Streptavidin-horseradish reagent was added, and then incubated at room temperature for 30 minutes. After 3 washings with wash buffer, 50  $\mu$ L TMB solution and hydrogen peroxide solution were added to each well and incubated at room temperature for 15 minutes. When adding 100  $\mu$ L stop solution, absorbance was measured at 450 nm on a spectrophotometer. Data were expressed as ng mouse osteocalcin in the medium per  $\mu$ g cell layer DNA content.

### Total protein

Cellular protein content was measured with a BCA protein assay kit (Beyotime, China). Briefly, 20  $\mu$ L cell lysate was mixed with 200  $\mu$ L working solution and incubated at 60°C for 30 minutes. The resulting optical densities were measured at 562 nm with a spectrophotometer. Bovine serum albumin was used to generate a standard curve.

### Total RNA extraction, cDNA synthesis and quantitative real-time RT-PCR analysis

Total RNA was isolated from osteoblasts with the RNeasy Mini kit (Qiagen, Germany). The amount and purity of RNA samples were measured by optical densitometry at 260 nm and 280 nm. RNA was converted into complementary DNA (cDNA) using a real-time polymerase chain reaction (RT-PCR) kit (Takara, Japan). Quantitative real-time RT-PCR was performed by Thermal Cycler Dice (Takara TP800, Japan). The sequences of primers for alkaline phosphatase-2 (AKP-2), osteocalcin (OC), collagen type I alpha1 (Colla1), and  $\beta$ -actin genes are given in Table I. Amplification reactions were performed with a SYBR PrimeScript RT-PCR kit (Takara, Japan). Ten microliters of SYBR Premix EX Taq was added to each well of an optical 96-well plate, 0.4  $\mu$ L of both forward and reverse

primer were added, as well as, 7.8  $\mu$ L ddH<sub>2</sub>O and 2  $\mu$ L cDNA sample. The plate was covered and centrifuged for several seconds to remove air bubbles, following PCR quantification using cycling parameters: 95°C, 10 seconds followed by 60°C, 34 seconds for 40 cycles. All samples were analyzed in triplicate. The comparative Ct-value method was used to calculate the relative quality of AKP-2, OC, Colla1, and  $\beta$ -actin. Expression of the housekeeping gene  $\beta$ -actin was used as internal control to normalize results.

### Statistical analysis

All statistical tests were carried out using SPSS (version 12.0; SPSS, Chicago, IL). All results were expressed as mean and standard deviation (SD) of 9 independent determinations ( $n = 9, 3 \times 3$ ) with 3 respective experiments, and tested for statistical significance with Student 2-tailed *t* test. *P* less than or equal to .05 was considered to indicate statistical significance.

## RESULTS

### Surface analysis of the discs

SEM images show similar patterns of surface topography of these 2 substrates. The surfaces are quite irregular for test and control group discs, and they both show a great increase in roughness (significant waviness) with porous network structure (Fig. 1). However, it seems that the numerous irregular micropits and indentations in the test surface (Fig. 1, A) are a little larger than those in the control surface (Fig. 1, B). The microscopic evaluation also demonstrates no residual particles from sandblasting on both surfaces.

X-ray diffraction demonstrates a titanium dioxide layer on the test surface and the crystal structure of this titanium dioxide is proved to be anatase (Fig. 2, test). Although there is no anatase on the surface of control disc, TiH<sub>2</sub> diffraction appears on the x-ray diffractometry pattern of the control surface (Fig. 2, control).

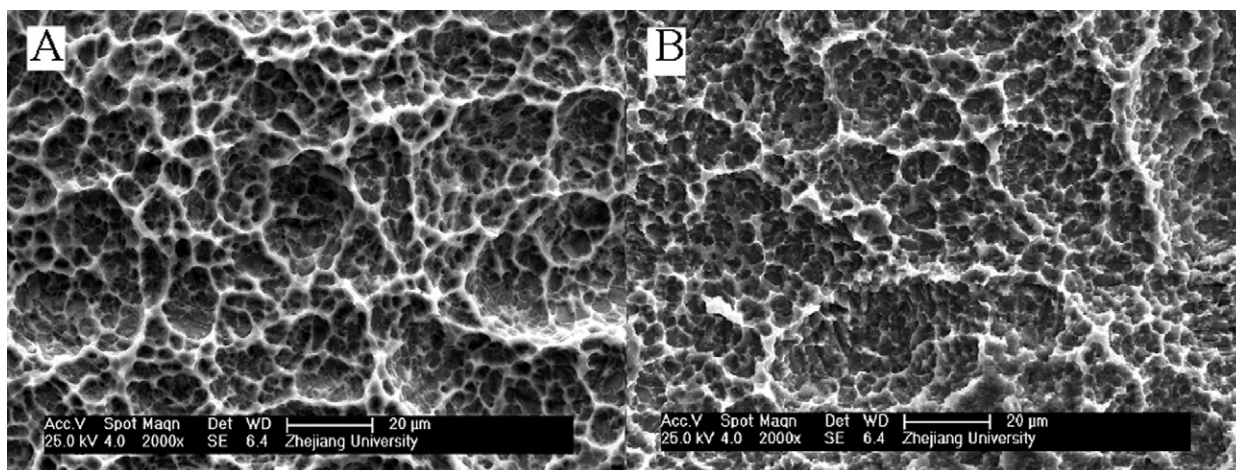


Fig. 1. SEM images of 2 different Ti alloy surfaces. **A**, Sandblasted, dual acid-etched and H<sub>2</sub>O<sub>2</sub>/HCl heat-treated Ti-6Al-4V disc (test group) has an irregular surface with porous network structure; **B**, Sandblasted, dual acid-etched treated Ti-6Al-4V disc (control group) has a similar surface, however, the micropits and indentations in this surface seem a little smaller compared with those in test surface.

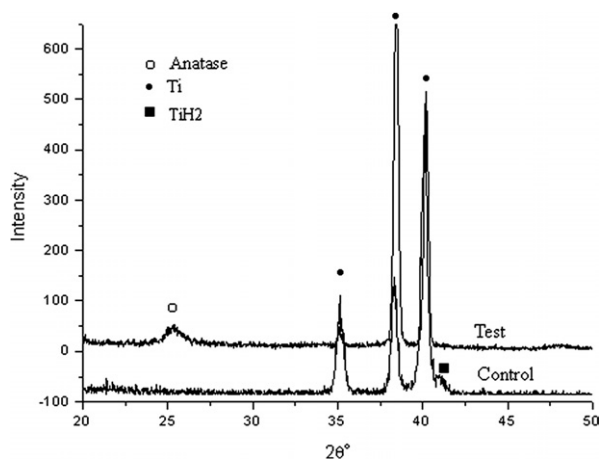


Fig. 2. XRD patterns of 2 different specimens. It shows anatase exists only in the surface of peroxide-treated disc (test), whereas TiH<sub>2</sub> exists in the surface of control group disc (control).

### Cell proliferation

Fig. 3 shows the mean DNA content in both groups, which reflects the number of MC3T3-E1 cells proliferated on Ti-6Al-4V discs. As it demonstrates, the DNA content was increased with culture time. At day 4, the DNA content was very low in both specimens, but it was largely enhanced at days 7 and 14. The DNA content in the test group was significantly higher than that in the control group at every time point investigated ( $P < .05$ ).

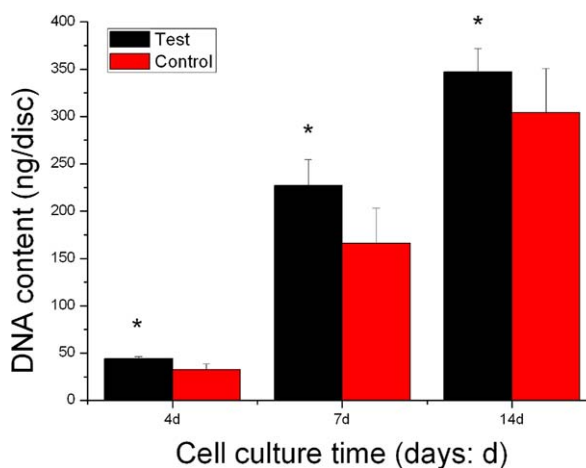


Fig. 3. Effect of Ti-6Al-4V surfaces on cell proliferation: cell proliferation was determined by fluorescence assay. The cellular DNA content was higher in test group than control group at all the time points. \* $P < .05$ .

### Osteogenic differentiation response

Fig. 4 summarizes cell-specific ALP activity level of MC3T3-E1 cells cultured in control and test groups in basic culture medium. A gradual increase in cell-specific ALP synthesis by cells cultured in both groups was observed with longer culture time. The test group demonstrated significantly increased cell-specific ALP activity level compared with the control group at every time point investigated ( $P < .05$ ).

The osteocalcin production released into cell culture medium at day 14 is shown in Fig. 5, and that has been

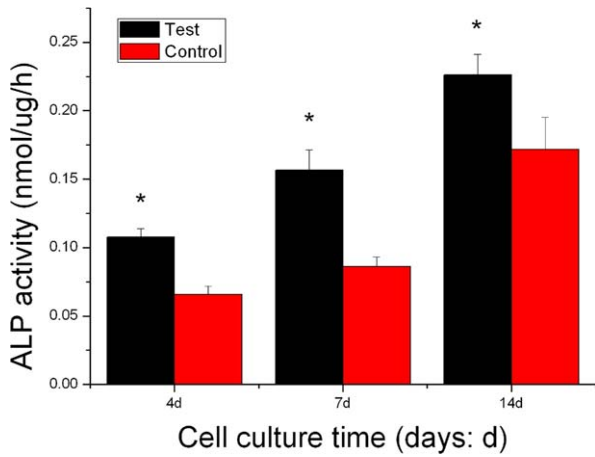


Fig. 4. The activity of intracellular ALP was much higher in test group than control group at every time point. ALP activity was normalized by total cellular protein. \* $P < .05$ .

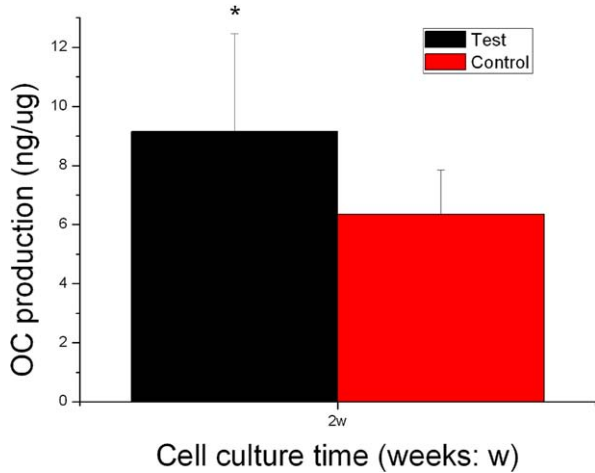


Fig. 5. Effect of different Ti-6Al-4V surfaces on osteocalcin production: osteocalcin content in culture medium was measured by EIA method. Data were expressed as total ng mouse osteocalcin/ $\mu$ g cell layer DNA. Cells in test group produced more osteocalcin than control group at day 14. \* $P < .05$ .

normalized by cell DNA content. The test surface significantly enhanced osteocalcin production compared with control surface ( $P < .05$ ).

Fig. 6 shows comparisons of mRNA transcript levels of bone-related genes of MC3T3-E1 cells cultured in control and test groups for 4, 7, and 14 days in the presence of basic medium. The expressions of AKP-2 mRNA and OC mRNA (Fig. 6, A, B) were very low at days 4 and 7 in both groups; however, expressions of these 2 mRNAs were significantly higher in the test group than in the control group ( $P < .05$  or  $P < .01$ ).

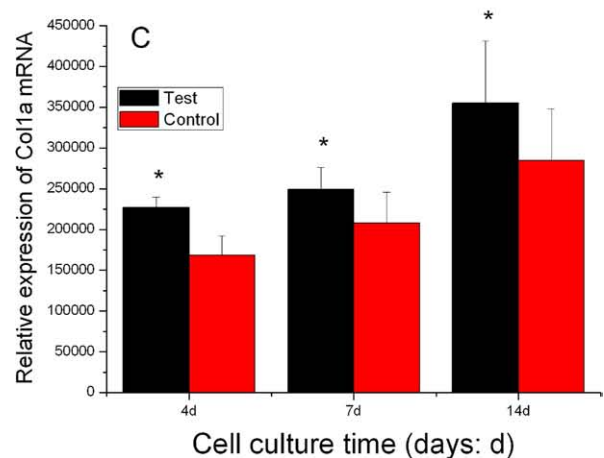
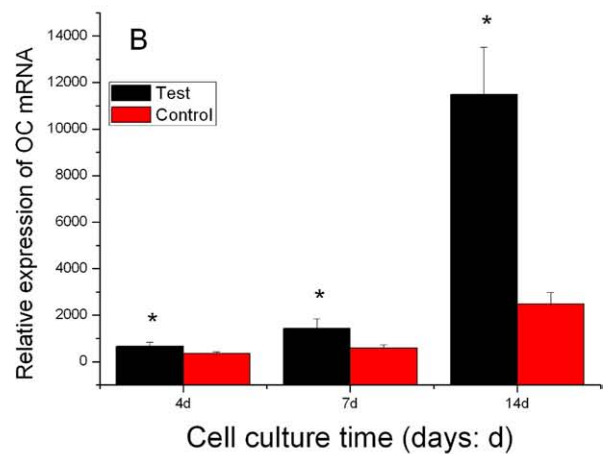
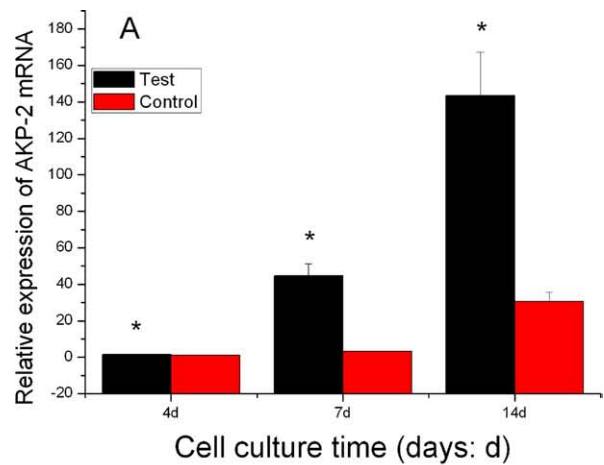


Fig. 6. Mean expression levels of AKP-2 mRNA (A), OC mRNA (B), and Colla1 mRNA (C) on different Ti-6Al-4V surfaces at days 4, 7, and 14. The transcriptional levels of the above 3 genes were significantly up-regulated in test group compared with control group. \* $P < .05$ .

At day 14, ALP mRNA and OC mRNA levels were very high, and were significantly up-regulated in the test group compared with the control group ( $P < .01$ ).

Regarding Colla1 mRNA expression, it revealed a tendency to increase in a time-dependent manner. As was indicated in Fig. 6, C, Colla1 mRNA expression was much higher in the test group than in the control group at all time points investigated ( $P < .05$ ).

## DISCUSSION

It is widely studied that the treatment with a  $H_2O_2/HCl$  solution at  $80^\circ C$  followed by heating at  $400^\circ C$  can produce an anatase titania gel layer on titanium surface, and that gel layer is able to induce apatite deposition when immersed in a simulated body fluid within several days.<sup>8,11,13,16</sup> However, few studies have been done to evaluate its bioactivity in vivo or in vitro. This study aimed to assess the bioactivity of this bioactive surface by examining proliferation and differentiation of pre-osteoblast cells in vitro.

The osseointegration of dental implants is important and osteoblastic cells play a critical role in the early stages of osseointegration. Many studies have assessed cell response to titanium surfaces in order to mimic the initial stages of osseointegration in vitro. MC3T3-E1 is one of the most widely used osteogenic cell lines among different cellular models, and it has proved to be a good candidate for studying cell attachment, proliferation, and differentiation of osteoblasts on titanium surfaces.

In our study, we found that cells seemed to proliferate much better on a test surface than on a control surface. Because these 2 surfaces share almost the same roughness, the difference in surface roughness might not be responsible for the great diversity in cell proliferation. Concerning the titanium hydride on the control surface, a previous report showed titanium hydride did not play an important role in bone response to sandblasting and etching (SLA) implant surface.<sup>19</sup> However, the effect of titanium hydride on osteoblast culture in vitro is not so clear. It has been confirmed that SLA surface contains a titanium hydride-rich layer,<sup>20,21</sup> and from numerous previous studies of the SLA surface, it might not be possible that titanium hydride inhibits cell growth. So, we believe that the enhanced cell proliferation might be due to the porous anatase formed on the rough surface, because this porous structure is important for adsorption of proteins and mineral ions, which is favorable for cell growth.<sup>22,23</sup>

ALP is up-regulated at an early stage of osteoblast differentiation, the activity of ALP is considered an early indicator of osteogenic differentiation, bone formation, and matrix mineralization.<sup>24,25</sup> In contrast, OC is secreted from mature osteoblasts and considered a late bone marker.<sup>24,26</sup> Collagen type I is a matrix protein synthesized by osteoblasts and during the latter stages of osteogenesis becomes mineralized with hy-

droxyapatite and also considered a bone marker.<sup>27</sup> In this study, we analyzed ALP activity and OC content after given culture periods, and found that ALP activity and OC content were significantly increased in test group compared with control group ( $P < .05$ ). Regarding AKP-2 mRNA, OC mRNA, and Colla1 mRNA expression levels, they were also all significantly higher in test group than in control group ( $P < .05$  or  $P < .01$ ). All these results suggest that this bioactive surface allowed rapid osteoblastic cell differentiation than control surface. Our result is in agreement with a previous report, which found titanium metal treated with hydrogen peroxide solution containing tantalum chloride could bond more tightly to bone than one that is not treated.<sup>12</sup>

It has been indicated that Ti surface chemistry, roughness, and topography affect osteoblastic cell adhesion, spreading, proliferation, and differentiation.<sup>28,29</sup> In the present study, test group specimens were further treated with  $H_2O_2/HCl$  and heating. Tengvall et al.<sup>30</sup> found that when Ti was treated with  $H_2O_2$  solution, a titania gel incorporated with superoxide radicals would form on the Ti surface. Wang et al.<sup>8</sup> suggested the superoxide radicals existed in the titania gel would disturb apatite nucleation. But a heat treatment,  $400^\circ C$  for 1 hour, might eliminate superoxide radicals, and thereby changed the titania gel's chemical properties and developed a porous Ti-O network that was suitable for apatite nucleation. In addition to porous Ti-O network, the anatase titania gel formed upon heating also played an important role in apatite deposition. Furthermore, the formation of Ti-OH groups when treated with  $H_2O_2/HCl$  solution could provide apatite deposition on Ti surface. Succinctly, this bioactive surface is favorable for apatite deposition, and it has been confirmed that osteogenesis was enhanced by growth of osteogenic cells on an apatitic surface.<sup>9</sup> Although osteogenic cells adhered to the surfaces of materials earlier than apatite formation, the ability for apatite deposition might be important in osteoblastic cell differentiation. From the previous reports and the present study, we found a similar surface structure was produced when Ti-6Al-4V and Ti were treated with  $H_2O_2/HCl$  and heating. Because Ti-6Al-4V and Ti share a similar surface chemistry when treated with  $H_2O_2/HCl$  and heating, the bioactive surface in the present study is supposed to be favorable for osteogenic cell differentiation, and our results have confirmed it.

Not only the surface chemistry of titanium alloy but also the surface roughness and topography may affect the function of osteogenic cells. Cell differentiation has been shown to be enhanced by surface roughness.<sup>31</sup> It has been demonstrated that surface roughness in the 1-

to 2- $\mu\text{m}$  range is favorable for biochemical anchorage of oral implants and shows stronger bone response than smoother or rougher surface.<sup>32-34</sup> In our study, the test surface control surfaces have similar moderately rough surfaces (between 1.0 and 2.0  $\mu\text{m}$ ), so surface roughness may not have contributed to the enhanced cell differentiation.

Chemical treatment of Ti-6Al-4V with  $\text{H}_2\text{O}_2/\text{HCl}$  is a simple method that has potential to improve bone formation. The treated Ti alloy surface has the ability to deposit apatite by itself to give osteoconductivity. Moreover, this simple method also has the advantage of forming bioactive porous titania gel layers on complicated surfaces.

In summary, chemical treatment including  $\text{H}_2\text{O}_2/\text{HCl}$  and heating provided this bioactive surface moderate surface roughness and porous titania gel layer, which might contribute to the significant enhanced proliferation and differentiation of MC3T3-E1.

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