ABSTRACT: The effect of changes in surface charge on the biological properties of implants is not clear. The objective of this study was to evaluate the biological properties of the surface of titanium sheets with different charges due to different treatment methods. Titanium sheets were sandblasted with large grit and underwent acid etching before being subsequently divided into the following groups: SLA, no further treatment; SLA-Ca²⁺, immersed in 1% CaCl₂ solution; SLA-NaCl, immersed in saline; and SLA-Ca²⁺-NaCl, immersed in 1% CaCl₂ solution followed by saline. Surface characteristics were evaluated using field-emission scanning electron microscopy with energy-dispersive spectrometry, surface profilometry, and contact angle assays. Additionally, we used a ζ-potential analyzer to directly measure the electrostatic charge on the different group surfaces. The effect of changes in the Ti surface on biological processes after different treatments was determined by analyzing fibronectin adsorption, osteoblast-like MG63 cell adhesion and proliferation, and the expression of osteogenesis-related genes. Compared to the SLA surface, the other three groups contained corresponding trace elements because they were soaked in different liquids; the contact angles of the three groups were not significantly different, but they were significantly smaller than that of the SLA group; and there was no change in the surface topography or roughness. Furthermore, the SLA-Ca²⁺ group had a significantly reduced negative charge compared to that of the other three groups. There were no differences between the SLA-NaCl and SLA-Ca²⁺-NaCl groups in terms of negative charge, and the SLA group surface carried the most negative charge. Fibronectin adsorption capacity and cytological performance testing further showed that the SLA-Ca²⁺ group had the most significant change, followed by the SLA-NaCl and SLA-Ca²⁺-NaCl groups; the SLA group had significantly lower capacity and performance than the other three groups. These results suggest that the surface charge of the titanium sheet changed when immersed in different liquids and that this treatment enhanced biocompatibility by reducing the electrostatic repulsion between biomaterials and biomolecules.

1. INTRODUCTION

It is well known that the surface characteristics of biomedical implants influence their interactions with cells and the extracellular matrix (ECM). Therefore, they are key factors in determining the osseointegration of implants. Modification methods commonly used on the surface of an implant affect the surface morphology, composition of chemical elements, molecular structure, charge state, surface free energy, and hydrophobic properties. These modifications aim to improve the biological properties of the implant surface and further affect the complex biological effects between the implant and the body. Most basic research focus, for example, on the effect of the morphological structure of the implant surface or the composition of chemical elements on the biological behavior of osteoblasts. There are no systematic studies on the impact of changes in the charge state of the implant surface on the interaction with proteins and cells. According to previous research by our team and related reports, the surface of titanium implants carries less negative charge after ultraviolet irradiation, weakening the electrostatic repulsion force against proteins, promoting protein adsorption, and playing an important role in the process of early cell adhesion; therefore, different surface charges affect the biological activity of the titanium surface. This interferes with the efficiency of early bone formation. As long as the sample is stored in an ordinary environment, its surface will be contaminated with hydrocarbons in a relatively short period of time, from the initial hydrophilic surface to a hydrophobic surface, the positive charge becomes negative, weakening the electrostatic repulsion between biomaterials and biomolecules.

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and the protein adsorption capacity also decreases. The early adhesion, proliferation, differentiation, and mineralization ability of osteoblasts is reduced to the level before UV irradiation; this phenomenon is called biological activity aging. More importantly, this pollution is almost inevitable in an ordinary environment. The cause of this biological aging may be related to the following aspects: as the sample is stored in an ordinary environment, the titanium surface gradually becomes contaminated with hydrocarbons. It is also related to the rapid disappearance of hydrophilicity and positive surface charge with storage time.  

In addition, the pH value of blood in normal humans is between 7.35 and 7.45. The isoelectric point of most proteins in the human body is less than 7. For example, the isoelectric point of fibronectin (Fn) is approximately 5.8, and bone morphogenetic Protein-2 (BMP2), which is important for bone formation, has an isoelectric point of approximately 4.8, which means that most proteins in the blood of normal humans are negatively charged (even the surface of osteoblasts is also negatively charged). Therefore, the positively charged (or negatively reduced) titanium surface irradiated by UV in the above study quickly forms early adhesions with negatively charged proteins through greater electrostatic attraction. Without treatment or aging after UV irradiation, the surface of the titanium implant carries a large amount of negative charge in the blood environment (our testing shows that the titanium implant carries a large amount of negative charge). Therefore, the positively charged (or negatively reduced) titanium surface irradiated by UV in the above study forms early adhesions with negatively charged proteins through greater electrostatic attraction. Without treatment or aging after UV irradiation, the surface of the titanium implant carries a large amount of negative charge in the blood environment (our testing shows that the surface charge of diﬀerent charge states was then examined using relevant cytological experiments.

2. RESULTS

2.1. Surface Characterization. Following previous manufacturing methods for SLA surface fabrication, microtopographies were successfully synthesized. Multilevel pores characterized by 10–30 μm pits and 1–3 μm micropits were formed on the specimen surfaces upon sandblasting with large grit and acid etching (Figure 1).

According to the energy-dispersive spectroscopy (EDS) test, very small amounts of Na, Ca, and Cl were found in the different groups. As shown in Table 1, except for the SLA group, the surfaces of the three groups of samples contained corresponding elements because they were soaked in diﬀerent liquids, although the content was very small. In addition, each group contains Ti and C because of pollution in an ordinary environment.

The $R_a$ values of the different groups were assayed, and the results are displayed in Figure 2. The $R_a$ values of the SLA,
NaCl, SLA-Ca\(^{2+}\), and SLA-Ca\(^{2+}\)-NaCl, respectively. The contact angles of the three groups immersed in different liquids had no significant difference, but they were significantly smaller than that of the SLA group, and the results are shown in Figure 4.

2.2. Surface Charge of the Specimens. \(\zeta\)-Potential measurements revealed a difference in electrokinetic interactions at the interface between the biomaterial surface and the aqueous electrolyte in this study. Figure 5 shows the \(\zeta\)-potential of the four different groups at pH 7.4. The SLA-Ca\(^{2+}\) group has the lowest absolute value of \(\zeta\)-potential compared to the other three groups. There were no significant differences in the contact angles between the SLA-NaCl, SLA-Ca\(^{2+}\), and SLA-Ca\(^{2+}\)-NaCl groups, but they were significantly smaller than that of the SLA group. **\(P < 0.01\)** compared with SLA-Ca\(^{2+}\), \(\&\&\) \(P < 0.01\) compared with SLA-NaCl, and \(\&\&\&\) \(P < 0.01\) compared with SLA-Ca\(^{2+}\)-NaCl.

2.3. Protein Adsorption. The protein adsorptive amounts of the SLA, SLA-NaCl, SLA-Ca\(^{2+}\), and SLA-Ca\(^{2+}\)-NaCl surfaces over different time periods were analyzed using a biocinchoninic acid assay, and the results are shown in Figure 6. The rate of protein adsorbed by the SLA-Ca\(^{2+}\) group was significantly higher than that of the other three groups at each time point. There was no significant difference between the rates of protein adsorbed by the SLA-NaCl and SLA-Ca\(^{2+}\)-NaCl groups, but these rates were significantly higher than those of the SLA group at each time point.

2.4. Cell Adhesion. Initial cell adhesion on the SLA, SLA-NaCl, SLA-Ca\(^{2+}\), and SLA-Ca\(^{2+}\)-NaCl surfaces was estimated by counting the number of MG63 cell nuclei stained with Hoechst dye, as shown in Figure 7. The results were calculated using Image-Pro Plus 6.0 software and are displayed in Figure 8. At each time interval, the number of adherent cells on the SLA-Ca\(^{2+}\) surfaces was dramatically higher than that on the other three surfaces \((P < 0.01)\). The cell number on the surface of the SLA-NaCl and SLA-Ca\(^{2+}\)-NaCl groups was significantly higher than that in the SLA group \((P < 0.01)\), but the two...
groups have no difference at each time point. At each incubation time point, the surfaces of the SLA group had the lowest number of cells among the groups.

2.5. Cell Proliferation. Cell proliferation was evaluated via the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay, as shown in Figure 9. Cell proliferation increased over time in all four groups. Cells grown on the SLA-Ca2+ surface had significantly higher proliferative activity than that on the other three surfaces (P < 0.01). The cell number on the surface of the SLA-NaCl and SLA-Ca2+-NaCl groups was significantly higher than that in the SLA group (P < 0.01), but the two groups have no difference at each time point. The surfaces of the SLA group had the lowest number of cells among the groups at each incubation time point. **P < 0.01 compared to the group of SLA-Ca2+, **P < 0.01 compared to the group of SLA-NaCl, and *&P < 0.01 compared to the group of SLA-Ca2+-NaCl.

2.6. Gene Expression Analysis. Gene expression on the different surfaces was quantified using real-time polymerase chain reaction (RT-PCR), as shown in Figure 10. Generally, high ALP expression was detected at week 1 and was subsequently greatly decreased at week 2. The ALP expression level in cells grown on the SLA-Ca2+ surface was higher than that in cells grown on other surfaces at the two time points (P < 0.01). The lowest level of ALP expression was observed in the SLA group (P < 0.01), but ALP expression was not significantly different between cells grown on the SLA-NaCl and SLA-Ca2+-NaCl surfaces at all time points. Similarly, RUNX2 expression was upregulated at week 1 and decreased to a low level at week 2. The group showing the highest RUNX2 expression level was SLA-Ca2+ (P < 0.01), and there were no significant differences among the other three groups at the two time points. For OCN, a low level of expression was observed in all groups at week 1, followed by upregulation during week 2. The groups with the highest and lowest expression levels of OCN, respectively, were SLA-Ca2+ and SLA groups at the two time points (P < 0.01). There was no statistical difference between the SLA-NaCl and SLA-Ca2+-NaCl surfaces at the two time points.

3. DISCUSSION

The ideal function of dental implant restoration is based on good osseointegration between the implant and bone tissue.11,14 The occurrence and formation of osseointegration include complex physiological processes involving mutual recognition, reaction, and a combination of artificial materials and organism hosts. When titanium implants and other biological materials are implanted into the host, they first recognize each other with proteins, fibers, ions, cytokines, and other components in the blood environment, leading to the adhesion, proliferation, and differentiation of osteoblasts.4,6 In addition to mineralization, osseointegration eventually forms.15 Therefore, the identification and attachment of proteins on the surface of implants and the adhesion of osteoblasts are key links in osseointegration. Rapid completion of the adhesion process is one of the requirements for the rapid realization of bone bonding.16 In the process of osteoblast adhesion, fibronectin is one of the main guide proteins in the ECM that mediates the osteoblasts to complete the adhesion step. Therefore, in this study, we used Fn as a model protein for our detection.
As the pH of normal human blood is approximately 7.4, and it is difficult to change this reality, only changing the charge state on the surface of the biological material can change the static electricity between the protein and the biological material in this environment. According to previous literature, researchers speculate that the surface of implants irradiated by UV carries positive charges and negatively charged proteins to quickly form early adhesion through electrostatic attraction and accelerates the enhancement of early osseointegration of implants.\(^{17,18}\) In our study, using the ZETA potentiometer, the surface of the titanium sheet after UV irradiation did not carry a positive charge, but it carried less negative charge than the control group.\(^7\) Therefore, the static electricity between the biological material and the protein, the repelling effect, was significantly weakened, which further improved the protein adsorption capacity of the titanium implant surface. This also significantly improves the biological performance of the implant material. However, aging after ultraviolet irradiation is inevitable. Therefore, in this study, we tried to change this condition by storing the implant in a liquid, so that the implant can be preserved for a long time and maintain good biological performance, at the same time avoiding hydrocarbon pollution in the air environment.\(^19\)

In this study, we prepared microrough surface morphology on the surface of a titanium sheet via large-particle sandblasting and acid etching technology. Through FESEM observations, we could observe typical primary and secondary pore structures. Forming a mechanical fit between the implant and the bone tissue to obtain good initial stability, the surface roughness test revealed that there was no difference between the groups immersed in different liquids. Examination of surface elements revealed that, except for the SLA group, the other three groups retained a very small amount of the corresponding elements on their surfaces because of immersion in different solutions. The titanium plates soaked in different solutions were removed and naturally dried at 25 °C for 30 min. Next, the hydrophilicity of the different samples was tested. This may be because some inorganic salts with hygroscopic effects remain on the surface of the samples or the samples were stored in the liquid to avoid the pollution of hydrocarbons, which resulted in the surface hydrophilicity of the three groups of titanium sheets preserved in their solutions being significantly better than that of the SLA group.

The ζ-potential is a parameter that reflects the state of charge between the solid and liquid interface. In this study, we used a solid surface ZETA potentiometer to detect the charge on the surface of each group of samples. The charge on the solid surface is affected by various factors, such as the surface chemical composition; the pH value of the surrounding environment can also affect the state of surface charge.\(^7\) Here, the pH value was set under normal physiological conditions to better simulate the real situation. According to our previous research, pure titanium has an isoelectric point of approximately 4.2; therefore, its surface carries a large amount of negative charge at a pH of 7.4. In addition, researchers have shown that the titanium implant surface is easily contaminated by hydrocarbons when stored under common conditions protected from light, which can lead to biological aging.\(^8−10\)

Our test results also confirmed that the SLA group carried the most negative charges. We used calcium ions carrying a bivalent positive charge as bridging ions. One positive charge is connected to the surface of the negatively charged titanium sheet, and another positive charge can adsorb negatively charged Fn (Figure 11). The surface charge detection results are as expected. After treatment with divalent calcium ions, the surface of the SLA-Ca\(^{2+}\) group generally carried the least negative charge; therefore, its surface Fn adsorption capacity was also the strongest. On the surface of the SLA-NaCl group, there are relatively few negative charges because part of the negative charge on the surface is neutralized with Na\(^+\) after the titanium sheet is soaked in saline. Similar to the SLA-NaCl group surface, the SLA-Ca\(^{2+}\)-NaCl group should carry the least negative charge after treatment with divalent calcium ions, but after being immersed in physiological saline, the positive charge on the surface is neutralized by Cl\(^−\). Therefore, the negative charges carried on the surface of the two groups were significantly reduced compared with the SLA group, and the surface of the SLA group generally carried the most negative charges. This result was verified again in the detection of the Fn adsorption capacity. The SLA-Ca\(^{2+}\) group with the least surface negative charge had the strongest Fn adsorption capacity. Both the SLA-NaCl and the SLA-Ca\(^{2+}\)-NaCl groups had similar Fn adsorption capacities, which were significantly higher than those of the SLA group, and the SLA group had the strongest electrostatic repulsion between the negative charge carried on the surface and Fn; therefore, its surface protein adsorption capacity was the weakest.

Although it has been reported that a hydrophilic surface selectively promotes the adsorption of fibrinogen,\(^3\) in the present study, the adsorption capacity of Fn on the surface of three groups of samples with the same hydrophilic effect was not completely consistent, which further explained the influence of surface charge on the adsorption capacity of Fn.

The biological performance of each group was verified by relevant cell experiments. In the early cell adhesion and proliferation experiments, we found that the early adhesion ability and cell proliferation of the cells in the SLA-Ca\(^{2+}\) group at each time point were significantly higher than those of the other three groups, followed by the SLA-NaCl and SLA-Ca\(^{2+}\)-NaCl groups, and the SLA group had the least adhesion and proliferation. This is consistent with the protein adsorption capacity of the surface of each group. We know that Fn plays a crucial role in the cell adhesion process. When Fn successfully adheres to the surface of the biological material, the cell adheres to the surface of biomaterials with the help of Fn.\(^20,21\)

The adsorption capacity of Fn further determines the early adhesion and proliferation of cells.

It is not clear whether the charge state of the implant surface affects the differentiation ability of the cells on the surface of each sample. In this study, the expression of osteogenesis-related genes (OCN, RUNX2, and ALP) by osteoblasts was studied by quantitative RT-PCR after 7 and 14 days of
incubation. Our results show that the SLA-Ca\(^{2+}\) surface generally elicited the highest osteogenic gene expression compared with the other groups. Second, the SLA-NaCl and SLA-Ca\(^{2+}\)-NaCl groups had the same expression level, while the expression levels of various osteogenic genes in the SLA group were almost the lowest at each time point. We believe that this is inseparable from the Fn adhesion ability and the cell adhesion proliferation ability of the titanium sheet surface.\(^{10,22}\) It should be particularly emphasized that there have been reports in the literature that calcium phosphate coating is attached to the surface of the implant through various techniques. The study found that implants with added calcium ions had improved cell adhesion, proliferation, and differentiation compared with the control group.\(^{23,24}\) In addition, some studies placed calcium phosphate-coated implants in simulated body fluids, and they found thatapatite crystals can be formed on the surface of the experimental group.\(^{25-27}\) The researchers speculate that this is related to the charge of calcium ions on the surface of the experimental group, but in these studies, the charge on the surface of each group of samples was not specifically detected.\(^{27,28}\) In this study, we further confirmed that the surface of the SLA-Ca\(^{2+}\) group carries the least negative charge, while the surface of the SLA-Ca\(^{2+}\)-NaCl group also contains a small amount of calcium ions. However, after incubation with NaCl, the positive charge carried by Ca\(^{2+}\) is neutralized, and the number of negative charges increases significantly. Thus, although its surface contains calcium ions, its protein adhesion ability and biological performance cannot reach the level of the SLA-Ca\(^{2+}\) group. This also confirms that surface charge plays a key role in improving the performance of biomaterials.

In this study, we emphasize the change in the surface charge of titanium. Immersing Ti in CaCl\(_2\) solution is a relatively simple method that elicits a certain effect. Of course, if other methods can be found to completely reverse the negative charge to a positive charge, then perhaps the protein adsorption capacity and cell biological performance will be further improved.

4. CONCLUSIONS

(1) When stored under common conditions protected from light, the SLA group carried the most negative charges because of contamination by hydrocarbons. By immersing the titanium sheet in the CaCl\(_2\) solution, the charge state on the surface is changed. The divalent calcium ion can be used as a bridge ion to adsorb the negative charge on the surface of pure titanium. Other divalent and even multivalent cations theoretically have the ability to act as bridge ions.

(2) After the surface charge state changes, the titanium sheet can adsorb more negatively charged proteins by bridging ions. For example, Fn plays an important role in cell adhesion and stretching. However, the influence of changes in surface charge on the conformation of proteins adsorbed on its surface requires further examination.

5. MATERIALS AND METHODS

5.1. Preparation of the Titanium Samples. Commercially available pure grade 2 titanium (Tengxing Metal Materials, Shenzhen, China) was cut into discs of 15 mm diameter and 1 mm thickness. All titanium surfaces were ground with silicon carbide sandpapers of 280, 360, 400, 600, and 1000 grit in series. The titanium specimens were then immersed in 10 mL of acetone (Fuyu, Tianjin, China) at room temperature, ultrasonicated for 20 min, ultrasonicated for a further 20 min in 10 mL of absolute alcohol (Fuyu), and then rinsed with deionized water. Subsequently, the specimens were dried at room temperature.

The titanium specimens were subjected to sandblasting with large grit and acid etching (SLA) and then divided into four groups. The first was stored at room temperature and away from light for 2 weeks (control group, SLA), the second was immersed in normal saline (pH 7) for at least 48 h (SLA-NaCl), the third group was immersed in 1% CaCl\(_2\) (pH 7) for at least 48 h (SLA-Ca\(^{2+}\)), and a subset of the third group was then immersed in normal saline for at least 48 h as the fourth group (SLA-Ca\(^{2+}\)-NaCl).

The SLA surfaces were prepared according to a previously described method.\(^{22,29}\) All titanium specimens were sandblasted with 120 μm Al\(_2\)O\(_3\) particles at a distance of 50 mm, at an angle of 90°. The air pressure used for blasting was 0.45 MPa, and the procedure was conducted for 30 s. Subsequently, the specimens were etched using a mixture of 18% (v/v) HCl and 49% (v/v) H\(_2\)SO\(_4\) at 60°C for 30 min and then ultrasonically cleaned in ddH\(_2\)O for 15 min. Finally, all specimens were air-dried at room temperature. All samples were collected, rinsed with deionized water, and sterilized via autoclaving before use. A 1% CaCl\(_2\) solution was degassed using a bacterial filter. Sterile saline was used for the injection.

5.2. Surface Characterization. Following previously described methods,\(^{24,30}\) the morphology of each sample was analyzed using field-emission scanning electron microscopy (FESEM; FEI-Quanta 400, Hillsboro, OR). The chemical composition of the samples was investigated via FESEM-EDS (FEI-Quanta 400, Hillsboro, OR) under vacuum conditions (~2 × 10\(^{-9}\) mbar). The surface roughness (R\(_s\)) was measured using a profilometer (Wyko NT9300; Veeco, NY). Water contact angle measurements were performed using the sessile drop technique (OCA40 Micro, DataPhysics, Filderstadt, Germany) at room temperature.

5.3. ζ-Potential. ζ-Potential data inferred from streaming potential were measured on four different titanium surfaces, applying a constant gap (0.1 mm) between two rectangular samples with the same surface treatment.\(^7\) The electrokinetic streaming potential (Anton Paar, Graz, Austria) was automatically surveyed in the forward and backward flow directions. The measurements were performed in 0.001 mol/L KCl solution (pH 11), and the pH was adjusted to 7.4 by addition of 0.1 mol/L HCl (pH 1) or 0.1 mol/L NaOH (pH 13). For statistical analysis, four streaming potentials were obtained at the same pH value.

5.4. Protein Adsorption Assay. Fn (Sigma-Aldrich) was used as the model protein. A Fn protein solution was prepared according to a classical protocol using phosphate-buffered saline (PBS) at pH 7.4. A 300 μL droplet of protein solution (5 μg/mL in Tris) was added to each of the titanium discs. After incubation for 0.5, 1, and 2 h under sterile humidified conditions at 37°C, the samples were transferred to a new 24-well plate and washed thrice with PBS. Next, 500 μL of 1% sodium dodecyl sulfate (SDS) solution was added to the wells, and the plate was shaken for 1 h at a constant speed on an orbital shaker (TS-100, Qilinbeier, Jiangsu, China) to detach proteins from the samples. An aliquot of 100 μL of the collected solution was mixed with 100 μL of micro-
bicinchoninic acid (Pierce Biotechnology, Inc., Rockford, IL) in a new 96-well plate and incubated at 37 °C for 1 h. The optical density (OD) of each disc was quantified using a microplate reader (680, Bio-Rad, Hercules, CA) at 562 nm. The rate of protein adsorption was calculated using a standard curve, obtained by plotting the average blank-corrected 595 nm reading for each bovine serum albumin (BSA) standard from the kit versus its concentration in μg/mL. The above tests were conducted with three parallel samples at each time point and repeated three times.

5.5. Cell Culture. A human MG63 osteosarcoma cell line was cultured in Dulbecco’s modified Eagle’s medium (DMEM; Hyclone, Thermo Fisher Scientific, Waltham, MA) supplemented with 10% (v/v) fetal bovine serum (Hyclone) and antibiotics (penicillin [100 U/mL] and streptomycin [100 μg/mL]) at 37 °C in a humidified atmosphere containing 5% CO₂. At 80% confluence, the cells were detached using 0.25% (w/v) trypsin in 1 mM ethylene diamine tetraacetic acid 4Na and seeded onto four different surfaces in 24-well plates at a density of 2.4 × 10⁴ cells/cm². The culture medium was replaced on alternate days. This study was reviewed and approved by the Ethics Committee of Stomatological Hospital, Southern Medical University, People’s Republic of China.

5.6. Cell Adhesion Assay. Cell attachment was initially evaluated by measuring the number of cells attached to the titanium substrates after 30 min, 1 h, and 2 h of incubation, as described previously. At each time point, nonadherent cells were removed by gentle rinsing with PBS. Adherent cells were fixed with 4% (w/v) paraformaldehyde for 30 min and then stained with fluorescent Hoechst 33342 dye for 5 min. Cell fluorescent microscopic images were recorded over an area of 1800 μm². Values representing the mean and the standard error of the number of attached cells were calculated from 10 different random fields from each disc using Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD; n = 3, a total of 30 fields of view for each group).

5.7. Cell Proliferation Assay. Cell proliferation was determined by measuring cell density on culture days 1, 3, 5, and 7 using tetrazolium salt-based colorimetry (MTS; Promega Corporation, Fitchburg, WI). At each time point, nonadherent cells were removed by gentle rinsing with PBS and transferred to a new 24-well plate. Next, 500 μL MTS reagent was added to each well and incubated with 100 μL MTS reagent at 37 °C for 1 h. The amount of formazan product was measured using a microplate reader at 490 nm.

5.8. Expression of Osteogenesis-Related Genes. The expression of osteogenesis-related genes was evaluated using RT-PCR as previously described. MG63 cells were seeded at 2 × 10⁴ cells/disc and cultured for 7 or 14 days. Total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific). One microgram of RNA from each sample was reverse transcribed into complementary DNA using the PrimeScript RT reagent kit (TaKaRa Bio, Otsu, Japan). The expression levels of osteogenesis-related genes, including ALP, RUNX2, and OCN, were quantified using an iQ5 Multicolor RT-PCR Detection System (Bio-Rad Laboratories Inc.) with SYBR Premix Ex Taq II (TaKaRa Bio). Data analysis was carried out using the IQ5 Optical System Software Version 2.0 (Bio-Rad Laboratories Inc.). The relative expression levels for each gene of interest were normalized to those of the housekeeping gene (GAPDH). The primers used are listed in Table 2.

Table 2. PCR Primer Sequences and Product Sizes (Base Pairs)α

| gene       | primer sequence                  | amplicon size (bp) |
|------------|----------------------------------|--------------------|
| ALP        | S: 5′-CATGCTGATGTGACACAGCAAAAGA-3′ | 141                |
|            | A: 5′-ACACGCAACTGGCGCTGTA3′       |                    |
| OCN        | S: 5′-GACGAGTTGGTCCAGACCA-3′      | 138                |
|            | A: 5′-CAACGGGAAGGAAAGAAGG-3′      |                    |
| Runx2      | S: 5′-TCCACACATTAGGGGACACAT-3′    | 136                |
|            | A: 5′-TGCTAATGCTTGTTGGTTCCA-3′    |                    |
| GAPDH      | S: 5′-TGCCACCCAGCAACATGGA-3′      | 186                |
|            | A: 5′-CTAATGCATAGTCGCCGCTAGGCA-3′|                    |

*PCR, polymerase chain reaction; ALP, alkaline phosphatase; RUNX2, runt-related transcription factor 2; and OCN, osteocalcin.

5.9. Statistical Analyses. The data were analyzed using SPSS 13.0 (SPSS Inc., Chicago, IL). Q-Q plots were used to test the data distribution. One-way analysis of variance followed by the Student–Newman–Keuls post hoc test was used to determine the level of significance. Factorial analysis was used to evaluate the effects of group and time. P-values < 0.05 were considered significant, and values of P < 0.01 were considered to be highly significant.

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Notes

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