Research Article

Icariin-Loaded TiO₂ Nanotubes for Regulation of the Bioactivity of Bone Marrow Cells

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To explore the effects of icariin on the biocompatibility of dental implants, icariin- (ICA-) loaded TiO₂ nanotubes were fabricated on Ti substrates via anodic oxidation and physical absorption. The surface characteristics of the specimens were monitored by field emission scanning electron microscopy (FE-SEM), X-ray diffractometry (XRD), contact angle measurements (CA), and high-pressure liquid chromatography. Additionally, the activities of bone marrow cells, such as cytoskeletal, proliferative activities, mineralization, and osteogenesis-related gene expression on the substrates were investigated in detail. The characterization results demonstrated that ICA-loaded TiO₂ nanotubes were successfully fabricated and the hydrophilicity of these TiO₂ nanotubes was significantly higher than that of the pure Ti groups. The results also showed that ICA-loaded TiO₂ nanotubes might not have enhanced effects on cell proliferation and ALP expression. However, it seemed to significantly promote differentiation of bone marrow cells, demonstrated by enhancing the formation of mineralized nodule and the upregulation of the gene expression such as OC, BSP, OPN, and COL-1. The results indicated that ICA-loaded TiO₂ nanotubes can modulate bioactivity of bone marrow cells, which is promising for potential applications in the orthopedics field.

1. Introduction

Bone marrow cells, a type of multilineage potential cell, can proliferate and differentiate into a variety of mesodermal lineages, such as osteoblasts [1], chondrocytes [2], and adipocytes [3]. In vivo, damaged bone tissue will recruit bone marrow cells from the surrounding bone marrow or peripheral circulation to participate in the osseointegration of bone-implant surfaces [4–7]. Therefore, preferentially inducing bone marrow cells differentiation toward osteoblast cells and further accelerating osteoanagenesis are imperative. Previous studies have suggested that the rate and extent of osseointegration are mainly determined by the properties of the implant surface [8]. TiO₂ nanotube (NT) modification of the surfaces of titanium substrates has been attracting increasing attention. Several investigators have revealed that the nanostructure influences the adhesion, differentiation,

and migration of bone marrow cells significantly [9–12] and that the fates of cells on NT arrays are size dependent [9, 13, 14], while the optimal diameter is still controversial. They found that cell adhesion and spreading are severely impaired on NTs with diameters larger than 50 nm [9, 15]. However, some previous studies confirmed that NTs with diameters of 70 nm can increase the bone-implant contact and osteogenesis-related gene expression [10]. Therefore, we employed NTs with diameters of 30 nm and 80 nm on the surfaces of Ti substrates for further study.

Previous studies have demonstrated that NT arrays could control the release of drugs, such as antibiotics and proteins, for hours [16]. Loading bioactive factors that can induce the adhesion and proliferation of bone marrow cells on titanium implants are the most common strategy to improve early osseointegration. Herba Epimedii is widely used as a complementary and alternative traditional Chinese medicine

for the treatment of osteoporosis in China. Icariin (ICA, $C_{33}H_{40}O_{15}$; molecular weight: 676.67) is the major flavonoid glycoside extracted from Epimedium and is considered the main active constituent. ICA may exert favorable osseointegration effects by enhancing the differentiation of MSCs into osteoblasts while inhibiting the adipogenesis of bone marrow cells [17] and can treat osteoporosis by increasing bone mineral content [18]. Recently, ICA has been widely applied for bone tissue engineering due to its safe, nontoxic, and osteoinductive properties as well as its low cost [19, 20]. The porous PLGA/TCP composite scaffold loaded with ICA was filled in mouse calvarial bone defects and induced significant new bone formation [21]. A recent study reported the generation of a novel bone repair scaffold consisting of a chitosan/nanosized hydroxyapatite system loaded with ICA that can control the release rate and extent of ICA and enhance bone repair. The *in vitro* bioactivity assay revealed that the loaded ICA was biologically active [22]. ICA increases bone marrow cells differentiation and bone mineralization most likely by upregulating the expression of NO synthesis, subsequently regulating Cbfa1/Runx2 [23, 24]. Meanwhile, ICA can inhibit bone resorption by reducing osteoclastic differentiation and induce osteoclasts apoptosis through an MAPKs/NF- κ B mechanism [25]. Owing to these advantages, ICA may have a potential application as an ideal osteogenesis agent in bone tissue engineering.

To the best of our knowledge, there have been few experiments studying the application of this Chinese drug on the osseointegration of titanium implants. Therefore, the purpose of this study was to evaluate the effect of ICA on bone marrow cells, which will provide evidence for further applications in the modification of dental implants.

2. Materials and Methods

2.1. Materials. Titanium disks (14 mm in diameter, 0.3 mm in thickness, and 99.9% purity) were supplied by the Northwest Institute for Nonferrous Metal Research (Xi'an, China). ICA (molecular formula, $C_{33}H_{40}O_{15}$; molecular weight, 676.67 Da) was purchased from the National Institute for the Control of Pharmaceuticals and Biological Products (Beijing, China).

2.2. Fabrication of TiO_2 Nanotubes. TiO_2 NT arrays were fabricated according to previously described procedures via an electrochemical anodization method [11]. Briefly, all the Ti disks were mechanically polished with 800, 1000, and 1200 grit silicon carbide paper. Each sample was then ultrasonically cleaned with acetone, ethanol, and deionized water for 15 min. The cleaned pure Ti disks served as the anodes and a platinum sheet served as the cathode. The electrolyte consisted of 0.5 wt% NH_4F and 3 vol % deionized water in ethylene glycol electrolyte. The anodization voltage was 10 V or 30 V for 2 h. Subsequently, the specimens were rinsed with deionized water and air-dried immediately. Finally, the NTs were sintered at 450°C for 3 h to crystallize the amorphous NTs into anatase structures. The morphologies of the titanium specimens were characterized using

a field emission scanning electron microscope (FE-SEM) (Nova Nano SEM430, Netherlands). An X-ray diffractometer (XRD) (LD1-Y-2000, China) was used to determine the surface phase compositions of the treated specimens. Contact angle (CA) measurements were performed using a contact angle goniometer (Data Physics, Germany) with 3 μ L of water on the surfaces.

2.3. Drug Loading. ICA was loaded onto the TiO_2 NT arrays via a simplified physical adsorption method. Briefly, an ICA solution of 0.5 mg/mL was prepared in ethyl alcohol supplemented with 0.1% dimethyl sulfoxide (DMSO, Sigma, USA) and stored at 4°C. The final DMSO concentration used in the medium was less than 0.01%. The NT substrates were cleaned with deionized water before the ICA loading. Subsequently, the specimens were fully immersed in the respective ICA solutions at room temperature for 30 min and then treated under ultrasonication for 5 min. After that, the surfaces of the titanium substrates were gently rinsed with PBS to remove the excess ICA and then lyophilized for 2 h.

2.4. *In Vitro* Release Assay. The ICA-loaded NT substrates were immersed in 3 mL of phosphate-buffered saline (PBS) solution (pH 7.4) in a 24-well plate with gentle shaking at 100 rpm at 37°C. At predetermined time points of 1, 6, 12, 24, 48, and 72 h, 500 μ L of PBS was extracted and replaced with fresh medium. A high-performance liquid chromatograph (HPLC) (Agilent Technologies 1260 Infinity, USA) was used to examine the amount of ICA released. The mobile phase was composed of a mixture of water/acetonitrile (65/35, v/v) and the flow rate was 1.0 mL per minute. A variable wavelength detector was used to detect the column effluent at 270 nm with a column temperature of 25°C.

2.5. Cell Culture. The animal protocol received approval from the Southern Medical University Animal Research Committee. The bone marrow cells were derived from two-week-old Sprague-Dawley rats. Briefly, SD rats were treated with deep anesthesia and cervical dislocation. Then, the bilateral femur and tibia were isolated aseptically, and the marrow cells were flushed out with Dulbecco's Minimum Essential Medium (DMEM, Gibco). After that, the cells were cultured in low-glucose DMEM supplemented with 10% fetal bovine serum (FBS, Gibco) at 37°C in a humidified atmosphere with 5% CO_2 . When reaching 80% confluence, the cells were detached with 0.25% trypsin (Sigma) and reseeded in new culture flasks. Bone marrow cells at passages 2–4 were used in this study.

2.6. Cytoskeleton Observation. Bone marrow cells were seeded onto the surfaces of titanium substrates (Ti, NT₁₀, NT₁₀/ICA_{0.5}, NT₃₀, and NT₃₀/ICA_{0.5}) at an initial density of 1×10^4 /well. After 24 h of culture, the cells were gently rinsed with PBS, fixed for 15 min in 4% paraformaldehyde, and then permeabilized in 3% Triton X-100 for 5 min. After that, the samples were stained with TRITC-Phalloidin (YEASEN, Shanghai, China) for 40 min to visualize the actin

TABLE 1: Primer sequences utilized for quantitative real-time PCR.

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
COL-1	GGAGAGAGCATGACCGATGG	AAGTTCCGGTGTGACTCGTG
OC	GGAGGGCAGTAAGGTGGTGAA	TCCTGGAAGCCAATGTGGTC
BSP	GAACGGGTTTCAGCAGACGA	GGGGCCTTCACTGGTGGTA
OPN	AGCATTCTCGAGGAAGCCAG	AGTGTTTGCTGTAATGCGCC
Runx2	AGCGGACGAGGCAAGAGTTT	GGGTTCTGAGGCGGGACA
β -actin	TGAAGTACCCATTGAACACGG	GGGTCATCTTTTCACGGTTGG

cytoskeleton and then counterstained with 4',6-diamidino-2-phenylindole (DAPI, Sigma, USA) to visualize the cell nuclei. Subsequently, the cytoskeleton and nuclei of the bone marrow cells were observed under an inverted fluorescence microscope (Olympus, Japan).

2.7. Cell Adhesion, Proliferation, and Mineralization. The initial attachment of the bone marrow cells was evaluated by quantifying the cells attached to the titanium surface. After culturing for 1, 2, and 4 h, the plates were gently rinsed with PBS to remove all nonadherent cells and then placed into new 24-well plates for assessment of cell numbers using the Cell Counting Kit-8 assay (CCK-8, Dojindo Molecular Technologies, Japan) in accordance with the manufacturer's instructions. For the proliferative assay, the cells were cultured on the plates for 1, 3, 5, and 7 d, and the cell numbers were measured using the CCK-8 assay.

Bone marrow cells were seeded on each sample at a density of 2×10^4 /well. After culturing for 7 and 14 days, the cell supernatants were collected and frozen at -80°C . An alkaline phosphatase activity fluorometric assay kit (Bio-Vision, Milpitas, CA, USA) was used to assess the ALP activity according to the manufacturer's instructions. The fluorescence intensity was measured at Ex/Em of 360/440 nm using a multimode reader (Spectra Max M5, US) and then the values of the ALP activities were calculated according to the standard curve.

Cell mineralization was assessed by Alizarin Red staining. Briefly, 2×10^4 cells per well were seeded onto each sample, and the mineralized solution was refreshed every two days. After culturing for 21 days, the specimens were washed three times with PBS, and then the cells were then fixed with 4% paraformaldehyde. After rinsing with deionized water, the specimens were dyed with 0.1% Alizarin Red solution (Cyagen, USA) for 30 min. Finally, the formation of mineralized nodules was observed under a stereomicroscope (SZX16, Olympus, Japan). The quantification of mineralization was performed as described previously [26]. Briefly, 10% cetylpyridinium chloride was added to each well to dissolve the deposition, and the solution was then collected. The absorbance at 562 nm was measured with a microplate reader.

2.8. Gene Expression Analysis. The expression of osteogenesis-related genes was analyzed using the reverse transcription polymerase chain reaction (RT-PCR). At 7 and 14 d of culture, the MSCs were rinsed with PBS, and the total RNA was extracted using TRIzol (Gibco, USA). Then, the RNA

was used in reverse transcription to generate cDNA using a Prime Script™ RT reagent kit (TaKaRa, Japan). PCR was performed using a real-time PCR kit (SYBR Premix Ex Taq II, TaKaRa) to detect osteopontin (OPN), osteocalcin (OC), bone sialoprotein (BSP), Runx2, and type I collagen (COL-I) mRNA on a Light Cycler 480 II (Roche, Germany). The primer sequences for the osteogenic genes are shown in Table 1. The housekeeping gene β -actin was used to normalize the relative expression quantities of target genes.

2.9. Statistical Analysis. All data are expressed as the mean $\pm$ standard deviation. Comparisons between groups were tested by one-way ANOVA, least significant difference (LSD) post hoc test (when the variance was regular), or Dunnett T3 test post hoc test (when the variance was irregular). The differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. The Characterization of the TiO_2 Nanotube Substrates. After anodization at 10 V and 30 V and annealing at 450°C for 3 h, the morphologies of the TiO_2 NT arrays were observed by FE-SEM. Before modification, the titanium surface was smooth with visible parallel polishing scratches as shown in Figure 1(a). However, the TiO_2 NTs fabricated with ethylene glycol displayed smooth walls and uniform diameters of approximately 30 nm and 80 nm for the 10 V and 30 V anodizations, respectively (Figures 1(b) and 1(c)). The result showed that the diameters of the TiO_2 NTs were positively correlated with the anodic oxidation voltage.

The XRD diffraction patterns of the TiO_2 NT array specimens before and after heat treatment are shown in Figure 2. The results indicated that the TiO_2 NT arrays before heat treatment contain crystalline Ti substrate peaks, and no anatase phase was detected (Figure 2(a)), while most of the TiO_2 NTs were transferred into the anatase phase by sintering at 450°C for 3 h (Figure 2(b)).

The TiO_2 NT substrates displayed significantly lower contact angles compared with pure Ti, which illustrated that the nanostructure increased the hydrophilicity of the Ti specimens, especially for NTs with 80 nm diameters, while the loading of ICA had little effect on the surface properties of the titanium substrates (Figure 3).

3.2. ICA Release Profile. The amount of ICA loaded onto the NT arrays was determined by the NT diameters. The $\text{NT}_{30}/\text{ICA}_{0.5}$ group accounted for approximately 7-fold more

FIGURE 1: FE-SEM images of the surfaces of Ti specimens before and after anodization. (a) Pure Ti, (b) NT arrays after anodization at 10 V (NT₁₀), and (c) NT arrays after anodization at 30 V (NT₃₀). TiO₂ NTs with different diameters can be fabricated with ethylene glycol, and the resulting diameter is positively related to the voltage of anodic oxidation.

FIGURE 2: XRD patterns of the NT substrates before and after calcination. (a) NT substrate before sintering and (b) NT substrate after sintering. The results showed that most of the TiO₂ NTs can be transferred from the amorphous to anatase phase after sintering.

ICA than the NT₁₀/ICA_{0.5} group. The total quantity of ICA released from the NT arrays was less than 10⁻⁵ M. Figure 4 shows that the NT₃₀/ICA_{0.5} group expressed a faster release time than the NT₁₀/ICA_{0.5} group. A large amount of the release occurred on the first day, and, after that, the release profiles were similar. The ICA released from the NT array groups decreased obviously at 3 d, and no ICA was detected by HPLC after that.

3.3. The Cell Morphology. To investigate cell behaviors on ICA-loaded NT array substrates, we observed the morphologies of bone marrow cells using an inverted fluorescence microscope (Figure 5). The cells adhered to the NT array surfaces spread out sufficiently with irregular or polygon shapes. Bone marrow cells cultured on small diameter NT arrays demonstrated well-spread morphologies with more lamellipodia, whereas the cells on large diameter NT arrays

displayed noticeable filopodium. Cells seeded on the surfaces of ICA-loaded NT substrates expressed more filopodium than nonloaded NT groups. In contrast, the cells on pure Ti substrates were partly inadequate with fewer protrusions.

3.4. Assay of Bone Marrow Cell Adhesion. CCK-8 assays were used to examine the adhesion rates of the experimental groups and the control group of Ti specimens. Figure 6(a) shows the adhesion of bone marrow cells on different substrates after culturing for 1, 2, and 4 h. The number of cells increased with the incubation time. Bone marrow cells cultured on the pure Ti and small diameter NT samples displayed comparatively higher adhesion abilities than the NTs with large diameters at 2 and 4 h ($P < 0.01$). These results show the adverse influence of the NTs with 80 nm diameters on the cytocompatibility. The loading of the ICA failed to significantly promote the early adhesion of cells.

3.5. Assay of Bone Marrow Cell Proliferation. The proliferation of the bone marrow cells seeded on the pure Ti and NT-modified substrates was measured using the CCK8 assay. After culturing for 1 d, there were no statistically significant differences among all of the groups ($P > 0.05$). On day 3, there were no statistically significant differences between the pure Ti and small diameter groups, while the viability of the cells on the NT₃₀ substrates was lower than on the untreated samples. However, the NT₃₀/ICA_{0.5} group increased the viability of the bone marrow cells compared with NT₃₀ without ICA loading. Overall, no experimental groups promoted the proliferation of cells compared with pure Ti at day 5. After culturing for 7 d, the rate of cell proliferation in the NT₃₀/ICA_{0.5} group was significantly higher than those of the Ti group and the NT₃₀ group, which may be attributed to the sealing of NT constructure by cells and the long-term additive effect of ICA. In summary, the NT array structure and the ICA loading yielded no prominent advantage on cell proliferation except on individual groups (Figure 6(b)).

3.6. Alkaline Phosphatase Activity of Bone Marrow Cells. The ALP activities of the bone marrow cells cultured on the

FIGURE 3: Images and the water contact angle results. The contact angles of the NT plates were lower than those of the Ti plates, and the contact angles decreased as the NT diameters increased, while ICA had no influence on the contact angle (^{###} $P < 0.001$ with Ti; ^{\$} $P < 0.05$ with the NT groups with the 10-V treatment).

FIGURE 4: The total release of ICA in the different treatment groups. The release profile confirmed that the TiO₂ NTs with large diameters can be loaded with more ICA, and the release time lasted 3 d in each group.

different titanium substrates were detected after culturing for 7 and 14 d (Figure 7). The results showed that there were no statistically significant differences between the pure Ti group and the experimental groups, while the NT₃₀/ICA_{0.5} group was significantly higher than the NT₃₀ group ($P < 0.01$) after culturing for 7 d. There were no significant differences in the

ALP activities between the pure Ti group and the experiment groups after culturing for 14 d.

3.7. Mineralized Nodule Formation. The Alizarin Red staining results of different specimens after 21 days of culture are shown in Figure 8. The mineralized area on the NT-treated surfaces was significantly larger than that on the pure Ti surface because many blank areas could be observed on the control group, whereas cell calcification occurred homogeneously on NT treated surfaces. The highest Alizarin Red staining of calcium deposition was observed on the NT₃₀/ICA_{0.5} groups, followed by the NT-treated group, whereas no significant difference was observed among the latter three groups. The results indicated that the calcium deposition on NT₃₀ group was enhanced by addition of ICA.

3.8. Osteogenesis-Related Gene Expression. The gene expression profiles for OPN, OC, COL-1, BSP, and Runx 2 mRNA at 7 and 14 d are shown in Figure 9. After culturing for 7 d, the NT₁₀ and NT₃₀ groups displayed statistically higher COL-1 levels than the pure Ti group ($P < 0.05$), while the loading of ICA failed to increase the expression of COL-1. The expression of OPN in the experimental groups was statistically higher than that of the pure Ti group, while no significant differences were detected among the experimental groups. NT₁₀, NT₃₀, and NT₃₀/ICA_{0.5} expressed higher levels of OC than that of the Ti group. The experimental groups of NT₁₀/ICA_{0.5}, NT₃₀, and NT₃₀/ICA_{0.5} upregulated the level of BSP, NT₃₀ was superior to NT₁₀, and NT₁₀/ICA_{0.5}

FIGURE 5: Fluorescence images of bone marrow cells after culturing for 24 h. (a) Ti, (b) NT₁₀, (c) NT₁₀/ICA_{0.5}, (d) NT₃₀, and (e) NT₃₀/ICA_{0.5}.

significantly increased the expression of BSP compared with NT₁₀. However, only the NT₁₀/ICA_{0.5} group upregulated the expression of Runx2 mRNA compared with pure Ti. The above results indicated that NT characteristics enhanced mRNA expression of OPN, OC, BSP, and COL-I. While ICA had no significantly positive effects on the expression of bone-related genes. After culturing for 14 d, the NT substrates significantly upregulated the expressions of BSP and OC. More importantly, NT₁₀/ICA_{0.5} further increased the expressions of both genes significantly compared with NT₁₀ groups, while there was no significant difference between NT₃₀ and NT₃₀/ICA_{0.5} groups. In addition, there were no significant differences in the expressions of COL-I, OPN, and Runx2 among the groups, with the exception of the NT₃₀ group (Figure 9).

4. Discussion

In this study, we fabricated TiO₂ NTs with different diameters and loaded them with ICA. The structures were expected to simulate the natural bone structure and promote the integration of implants with the surrounding bone tissue.

TiO₂ NTs have attracted great interest due to their simple preparation process of anodic oxidation, low costs, and adjustable resistances. The diameters of the TiO₂ NTs were positively correlated with the anodic oxidation voltage. In this study, even and uniform TiO₂ NT arrays with diameters of approximately 30 nm and 80 nm were formed on the titanium plates. The results fully demonstrated the diversity and controllability of the TiO₂ NT structures; these properties are consistent with previous studies [15, 27, 28]. These studies

FIGURE 6: Adhesion and proliferation of bone marrow cells on the different surfaces. (a) The average adhesion of the cells on the surfaces of the NTs and the untreated Ti after culturing for 1, 2, and 4 h. (b) The proliferation of the cells on the specimens after culturing for 1, 3, 5, and 7 d (* $P < 0.05$ with Ti; *** $P < 0.001$ with Ti; \$\$ $P < 0.01$ with small diameter NT array specimens; ### $P < 0.001$ between the NT₃₀ and NT₃₀/ICA_{0.5} groups).

FIGURE 7: ALP activities of the MSCs on the different surfaces after culturing for 7 and 14 d (&& $P < 0.01$ between the NT₃₀ and NT₃₀/ICA_{0.5} groups). The NT₃₀/ICA_{0.5} group exhibited upregulated ALP activity after culture for 7 d compared with the NT₃₀ group, whereas no significant difference was observed with the other groups, which showed that ICA was slightly beneficial for ALP activity.

found that the bioactivities of cells were decided by the TiO₂ NT diameter. Park suggested that a smaller diameter of 15 nm could promote the proliferation and differentiation of osteoblasts, while large diameters of more than 50 nm caused serious damage to the cells due to the diverse surface morphologies [13, 15]. However, large diameter titanium NTs (70 nm) had advantages compared with small diameter NTs

in promoting the osteoanagenesis and increasing bone mass around an implant in vivo [10]. Because the optimal diameter of TiO₂ NTs has not reached a consensus, we fabricated TiO₂ NT specimens with two different diameters (30 nm and 80 nm) for subsequent research. TiO₂ NT plates were sintered at 450°C for 3 h. XRD showed that the amorphous TiO₂ changed into the anatase phase after sintering. It has been reported that anatase TiO₂ NTs have improved cell activity due to the similarity in the crystal lattice of the anatase structure and hydroxyapatite [29].

The physical and chemical properties of the titanium surfaces have an effect on the biological characteristics of the cells due to the direct contact with the surfaces of the implants. Rupp et al. [30] took advantage of a dynamic contact angle analysis system to monitor the hydrophilia of titanium specimens and found that the hydrophilia could be greatly affected by the roughness and morphology of the titanium surface. In this study, the water contact angle was used to measure the hydrophilia of various titanium specimens. The contact angles of the TiO₂ NT substrates were significantly lower than that of the pure Ti. Takebe [31] indicated that the hydrophilia of the TiO₂ NT structures may have increased greatly because the water easily infiltrated into the TiO₂ porous network, which caused the contact angle to decrease. In addition, the ICA loaded onto the NTs failed to influence the surface properties of titanium substrates.

Physical adsorption as a method to load the bioactive molecules and drugs onto the biomaterial scaffolds has been widely applied in previous studies [32–34]. This method has the advantages of ensuring the chemical structure and biological properties of the molecules, except for the inexpensive and efficacious characteristics. Here, the HPLC results showed that the ICA released from the NT structure in a

FIGURE 8: Alizarin Red staining and calcium deposition of bone marrow cell culture on various surfaces for 21 days. (a) Ti, (b) NT₁₀, (c) NT₁₀/ICA_{0.5}, (d) NT₃₀, and (e) NT₃₀/ICA_{0.5}. The formation of mineralized nodules was slightly enhanced in NT₃₀/ICA_{0.5} group (* $P < 0.05$ between the Ti and NT₃₀/ICA_{0.5} groups).

sustained manner, which indirectly confirmed the successful loading of ICA onto the NT surface and retention of the activity of the medicine. NT substrates as drug nanoreservoirs may represent a potential drug delivery system and the absorbing capacity was primarily dependent on the nanotube size and concentration of a drug, [35]; thus more drug was loaded with increasing diameter of NTs.

The initial adhesion of cells on the titanium substrates played an important role in the regulation of potential cell functions including proliferation, migration, and differentiation. Overall, large diameter NT arrays had adverse effect on the adhesion and proliferation of bone marrow cells compared with Ti and small diameter NT groups. Previous studies have suggested that the effects of TiO₂ NTs with various diameters on the behaviors of cells were more important than the surface topographies of the titanium substrates, which is consistent with the results of this study [36]. The inhibition of cell proliferation by NTs with large diameters may be related to differentiation due to the reciprocal relationship between cell proliferation and differentiation [37]. The effects of ICA on the proliferation of cells were dose-dependent: concentrations higher than 10⁻⁴ M are toxic to cells, and no positive effect on cell proliferation was detected even in lower dose group, which was consistent with the present study [38]. In this study, the quantity of ICA released from the NTs was less than 10⁻⁵ M. In addition, the bone marrow cells seeded on the small diameter TiO₂ NTs extended many lamellipodia, while more filopodia were observed on the surfaces of the

large diameter NT and ICA-loaded specimens. These results were also confirmed in previous studies [9]. Some scholars suggested that the filopodia may be generated to block the NT structures from the cells. The extracellular matrix secreted by osteoblasts was deposited on the surfaces of the NT walls with large diameter, which affects the cell adhesion [39].

In this study, the ALP activities of the MSCs adhered to the different substrates were assessed. This property is commonly used to indicate the early stages of osteoblast differentiation. After culturing for 7 d, the ICA-loaded NT₃₀ substrates displayed advantages over the NT and pure Ti substrates. The potential mechanism may be that the released ICA promoted the differentiation of the MSCs into osteoblasts. There were no significant differences among the groups at day 14. The phenomenon may be explained by the fact that the MSCs had entered into the late stage of differentiation and reduced the sensitivity to the topography of the titanium surface. On the other hand, the ICA was fully released from the NTs. The accumulated evidence proved that the NT structure could improve the ALP activity of the primary osteoblasts and cell lines [40], which was not in agreement with the results of this study and the concrete mechanism remains to be further studied. Mineralized nodule formation served as a marker of late stage differentiation, which is an essential sign for the osteogenic differentiation of bone marrow cells. Therefore, the above described results verified that ICA, particularly on the NT₃₀ surface, can significantly enhance the mineralization of bone marrow

FIGURE 9: Expressions of bone-related genes in bone marrow cells on different substrates for 7 d and 14 d (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ with Ti). NT and ICA-loaded NT groups exhibited significantly increased gene expression compared with the pure NT arrays.

cells, which was consistent with previous reports that ICA can promote the mineralization of cells [41].

We then investigated the molecular basis of Ti samples by assessing the mRNA expression of osteoblast-related genes, including OPN, OC, BSP, COL-I, and Runx2, which are important indices of bone maturity. Previous studies confirmed that BSP, OC, and COL-I are related to the cell cycle and regulate generation of the extracellular matrix and thus play an indispensable role in osteogenesis [42–44]. A previous study found that ICA displayed osteogenic capability to activate the PKG signaling pathway, further regulating the transcription of downstream osteogenic genes, such as COL-I, OC, and BSP [45]. After incubation for 7 d, the osteogenesis-related genes, such as OPN, OC, BSP, and COL-I, were upregulated by NT groups compared with the Ti group. We attributed the upregulation of the genes to the special nanostructures. Oh et al. [9] proposed that TiO₂ NTs with large diameters (70–100 nm) could selectively induce the differentiation of stem cells. BSP and OC are the most closely related to bone marrow cell differentiation [43]. A previous study demonstrated the effect of PLGA/TCP/icariin scaffold on upregulating BSP mRNA expression in dose-dependent manner. The mRNA expression level of OC in the icariin treated groups was higher than that in the control group [21]. In the present study, the ability of ICA to upregulate the levels of BSP and OC mRNA expression indicated that ICA is conducive to improving the osteogenesis potential of the bone marrow cells.

Taken together, the results indicate that both ICA and TiO₂ nanotubes are beneficial for improving the expression of osteogenesis-related genes in vitro, resulting in favorable molecular responses. However, the osteogenesis capability of ICA was weaker than TiO₂ nanotubes.

5. Conclusion

In this study, NT array specimens were fabricated and it was beneficial for controlling the release of ICA. NT array substrates facilitate the initial spreading, mineralization, and expression of bone-related genes of bone marrow cells, indicating a potential application on Ti implant. In addition, ICA significantly promoted the osteogenic differentiation in the later stage, such as enhancing the formation of mineralized nodule and upregulating the gene expression of BSP and OC. While it had no positive effect on cell bioactivity, thus the potential application of ICA as a substitute bioactive molecule on Ti implants should be further modified.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Supplementary Materials

Schematic illustration of the fabrication of icariin-loaded NT substrates and cellular responses. (*Supplementary Materials*)

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