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Preparation of icariin/TiO_2 nanotube composite coating on pure titanium surface and analysis of early drug release

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Abstract. – OBJECTIVE: The surface modification of pure titanium and the improvement of the early osseointegration of titanium implants are research hotspots currently. This study aimed to load icariin onto the surface of TiO_2 nanotube to form the composite coating, and to study the amount of loaded drug and the importance of early release.

MATERIALS AND METHODS: The TiO_2 nanotube was formed on the surface of smooth pure titanium *via* anodic oxidation, and both smooth pure titanium and TiO_2 nanotube were characterized using the scanning electron microscope, atomic force microscope and contact angle apparatus. Moreover, icariin was loaded onto the surface of pure titanium and TiO_2 nanotube using the soaking method.

RESULTS: The amount of early drug release in both types of materials was determined *via* high-performance liquid chromatography, the amount of loaded drug was calculated, and the curves of cumulative release amount and cumulative release percentage were plotted. The TiO_2 nanotube had a diameter of 70 nm with greater roughness than that of pure titanium ($p<0.05$) and smaller contact angle than that of pure titanium ($p<0.05$). The cumulative drug release amount in the first 4 d and the curve of cumulative drug release in the first 4 h in icariin/TiO_2 nanotube group were significantly larger and higher than those of the icariin/pure titanium group. The curve of cumulative drug release percentage was flatter and the release time was longer in icariin/TiO_2 nanotube group than those in the icariin/pure titanium group.

CONCLUSIONS: Our results indicate that the icariin/TiO_2 nanotube composite coating can provide a higher drug loading amount with a sustained-release effect.

Key Words
Pure titanium, TiO_2 nanotube, Icariin, Osseointegration, Characterize, Drug loading, Soaking method, Drug release, Sustained release, NSFC.

Introduction

Due to excellent mechanical strength, resistance to corrosion and biocompatibility, titanium has become a preferred material for implants[1]. However, titanium is a biologically inert material, so it fails to form decent early osseointegration with surrounding tissue. In addition, the osseointegration process of implants can be slowed down by some metabolic diseases, leading to the failure of implant placement[2]. Therefore, improving the early osseointegration of implants through surface modification of titanium is a research hotspot for scholars. At present, the methods for surface modification of titanium implants mainly include mechanical method[3,4], chemical method[5], coating modification method[6] and anodic oxidation method[7]. The first two of which can form the ultra-micro structure and chemical surface benefitting osseointegration on the surface of pure titanium (PT), but cannot form the controllable surface topography. Residues are easily produced in the operation, thereby affecting the adhesion, proliferation, differentiation and other functions of osteoblasts and bone mesenchymal stem cells. Coating modification method is to form the coating by loading the biological and chemical molecules or drugs with the osteogenic ability onto the surface of PT, to promote the osseointegration ability around implants. However, the bonding strength between the coating and surface of PT is poor, and the coating will shed off easily, thus affecting its osteogenic effect. The limitation associated with the abovementioned methods were overcome in the anodic oxidation method, and the uniform and controllable surface topography of TiO_2 nanotube (NT) can be formed. In addition, the structure of NT is similar to human bone tissue, which has good biocompatibility, and its high specific surface area can effectively promote the

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adhesion, climbing, growth and differentiation of osteoblasts and bone mesenchymal stem cells[8,9]. Icariin is a traditional Chinese medicine with promising prospect among the osteogenic drugs, which can regulate a variety of signaling pathways to promote osteogenesis and inhibit bone destruction[10,11] with a definite effect of osteoinduction in the culture of pre-osteoblasts *in vivo* and animal model of osteoporosis *in vitro*[12,13]. Loading icariin onto the surface of implants is expected to accelerate the early osseointegration process of implants[14]. The osteogenic drug was loaded onto the surface of NT to form the icariin/NT composite coating. NT has a descent bearing capacity and can keep the loaded icariin firmly and increase the osteogenic feature of NT and icariin. Currently, with the gradual expansion of research on the drug-loaded coating of implants, the drug release property has attracted more scientific attention. Osseointegration after implant placement is a sustaining process, in which the sustained drug release from the drug-loaded coating of implants is required and the sufficient drug concentration must be provided for tissues around implants[15]. Therefore, detecting the drug-loading amount of icariin/NT composite coating and studying its rule of sustained r[illegible] are pivotal in the study on its effect of early [illegible]oinduction. In this work, icariin was loaded o[illegible] the surface of NT to prepare the com[illegible]e coatin[illegible] and the drug-loading amount wa[illegible]te[illegible]. Also, the drug release curve was ob[illegible]ed, an[illegible]e rule of early drug release was st[illegible]. Th[illegible] of this study provided an e[illegible]imen[illegible]sis for the application of icariin/N[illegible]omposite [illegible]ing in the surface modificatio[illegible]im[illegible]ts.

Material and Methods

Design

[illegible] grou[illegible]d comparative observa-t[illegible].

Time and Place

Th[illegible]aterials were prepared in the Central Laborat[illegible]of School of Stomatology of Tianjin Medical University (Tianjin, China) from April to June 2015. The materials were characterized in the Hebei Key Laboratory of New Functional Materials (Tianjin, China) from July to August 2015. The drug was released in the Department of Pharmacology of Tianjin Medical University from August to October 2015. The main materials and equipment are shown in Table I.

Table I. Main materials and equipment.

Main materials and equipment	
Materials and equipment	Source
PT	Northwest Institute for Nonferrous Metal Research
Icariin	Sigma-Aldrich, St. Louis, MO, USA
High-voltage direct-current power supply	Tianjin Dongwen[illegible] Power Suppl[illegible]
Electromagnetic stirrer	Jiangsu Jin[illegible]engxiXiao[illegible] Electronic[illegible]
Platinum electrode	Tiani[illegible]HDAhe[illegible]ng Sci[illegible] T[illegible]ology Dev[illegible], Ltd.
Scanning electron microscope (SE[illegible]	[illegible]achi[illegible]an
Atomic force microsco[illegible]M)	Agi[illegible]USA
Contact [illegible]gle app[illegible]tus	Xiamen C[illegible]ongda Intelligent Technology Co., Ltd.
[illegible]performance [illegible]quid [illegible]hromatogra[illegible]strument	[illegible]nt, USA

Main Material and Instruments

Pure titanium (Northwest Nonferrous Metal Research Institute, China), Icariin (Sigma-Aldrich, St. Louis, MA, USA), High-voltage DC power supply (Tianjin Dongwen High Voltage Power Supply Factory, China), Electromagnetic stirrer (Jiangsu Jintan Xixiaoyang Electronic Equipment Factory, China), Platinum electrode (Tianjin Aida Hengsheng Technology Development Limited Company, China), Scanning electron microscope (Hitachi, Tokyo, Japan), High performance liquid chromatography (Sigma-Aldrich, St. Louis, MA, USA).

Experimental Method

Pretreatment of PT

A total of 40 round titanium plates with a diameter of 1.5 cm were ground and polished progressively using the silicon carbide sandpaper to obtain the 600 mesh. After that, plates were treated with ultrasonic cleaning using the acetone, absolute ethanol and deionized water for 10 min, and plated were then naturally dried. The treated samples were randomly divided into a smooth PT group (n=20) and NT group (n=20).

Preparation of NT Sample

Samples in the NT group were connected to the anode, while platinum was connected to the cathode, and they were placed in the hydrofluoric acid (HF) electrolyte in a concentration of 0.34% and constantly stirred in an electromagnetic stirrer. After the anodic oxidation under the high-voltage (18 V) direct-current power supply for 40 min, the NT was formed on the surface of samples, followed by ultrasonic cleaning with deionized water for 20 s. Finally, samples were dried for standby application.

SEM (Scanning Electron Microscope) Observation

5 plates were randomly selected from both groups. A thin layer of palladium membrane was formed on the surface of samples via sputter coating, and the surface topography of samples was collected and recorded *via* Scanning Electron Microscope (SEM) under an accelerating voltage of 5 kV.

AFM (Atomic Force Microscope) Detection

The three-dimensional structure and r metric roughness of samples were character d *via* Atomic Force Microscope (AFM). 10 pla were randomly selected from the tw ups. Th nanoprobe contacted the sample a anning rate of 2 Hz in the 1×1 μm² sca ng are nd the average was taken as the na on c r samples in each group.

Contact Angle D ect

5 plates we randomly ected from both groups. 10 μL deionized w was added at 5 cm abov surf of samples, and the contact angle (ei ed w ter on the surface of sampl was m dured u g a contact angle apparatus reflec hydrophilicity of samples. T angle as det d 10 times for each sample and ge was taken to represent the co ngle va e of the sample. The average in each g p represented the contact angle value in each g up.

Loading of Icariin on the Surface of Material

PT is widely recognized as a kind of biological material with high biosecurity, which is considered to be non-cytotoxic with wide application in the field of oral implantation[1]. It is needed to consider the cytotoxicity of icariin as a human implant material. The preliminary experiment in this study showed that the icariin solution in a concentration of 10^{-5} to 10^{-9} had no significant cytotoxicity activity against osteoblasts and bone mesenchymal stem cells, which was consistent with the results in previous studies[16,17]. In the preliminary experiment, the concentration of drug release in the icariin/NT composite coating formed by soaking in the icariin in c ncentration of 2×10^{-3} mol/mL was also d te d the results revealed that the conc ation of iin was 10^{-5} to 10^{-8} during the w process e-lease from 1 d to 14 d. T efore olution ith a concentration of 2× mol/mL d in this work. Icariin loade sing t soaking method, and the pr ed ariin/NT composite coating had hi safet this s y, icariin was prepared i e solutio oncentration of 2×10^{-3} m L g dimeth ulfoxide (DMSO), and samples in t T group (n=5) and the NT gr 5) were s d in icariin solution for 2 and then air dried for 1 d. The surface was c kly wash with 500 μL of Phosphate-Buffer Saline (P ; Gibco, Grand Island, NY, USA) abs pipette, and the excess icariin without binding to the smooth titanium plate and NT ly was removed. The PT loading icariin (PT-ICA) group and the NT loading icariin (NT-ICA) group were set up.

Detection of Icariin Loading Amount and Rule of Early Release

After washing, samples in the PT-ICA group and NT-ICA group were placed into a 24-well plate and soaked in 500 μL of PBS, followed by water bath on a shaking table at 50 rpm and 37°C. In the first 4 h, the soaking solution was discarded every other hour. 500 μL of fresh PBS was added, and the concentration of icariin in soaking solution was detected *via* high-performance liquid chromatography. Then, PBS was replaced every other day until all icariin was released into PBS. The average of release amount at different time points in the PT-ICA group and NT-ICA group was calculated; the loading amount was also calculated, and the curves of cumulative release amount and cumulative release percentage were plotted.

Main Observation Indexes

Detection results of SEM, AFM and contact angle on the surface of the sample, and detection results of icariin loading amount and rule of early release.

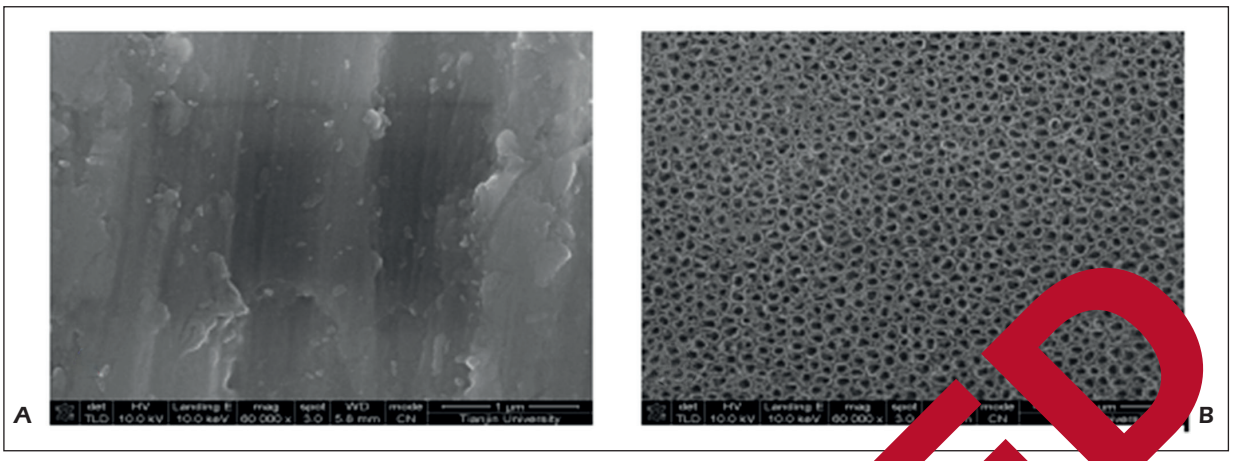

Figure 1. SEM observation of surface topography of samples in both groups. **A**, PT group (**B**, N group (×60k). **A**, Surface topography of smooth PT: no special structure on the surface. **B**, Surface topography of N distribut bular structure on the surface.

Statistical Analysis

Statistical Product and Service Solutions 12.0 (SPSS Inc., Chicago, IL, USA) software was used for the paired *t*-test. $p<0.05$ suggested that the difference was statistically significant.

Results

SEM Results

No special structure was dete he surface of smooth PT samples (Fi e 1A) he uniform and vertically-distrib bul was formed on the surf of N ples after anodic oxidation, the diameter about 80 nm, the tube wall th he as about nm and the tube gap was roximat 0 nm (Figure 1B).

AFM Analysis

hree-dime nal view of AFM is s wn in Figure 2, and it can be seen that there only the atch caused by mechanical polis on the face of PT (Figure 2A), and convex nanometric structure on the surface of NT (Figure 2B). According to our analysis, roughness in the NT group (424.6±20.4) was arg than that in the PT group (183.4±13.8) (Table II; $p<0.05$).

Analysis of Detection Results of Contact Angle

The contact angle in the NT group [(15.8±4.2)] was smaller than that in the PT group [(46.3±5.4)] ($p<0.05$; Table II), and the hydrophilicity in the NT group was higher than that in the PT group.

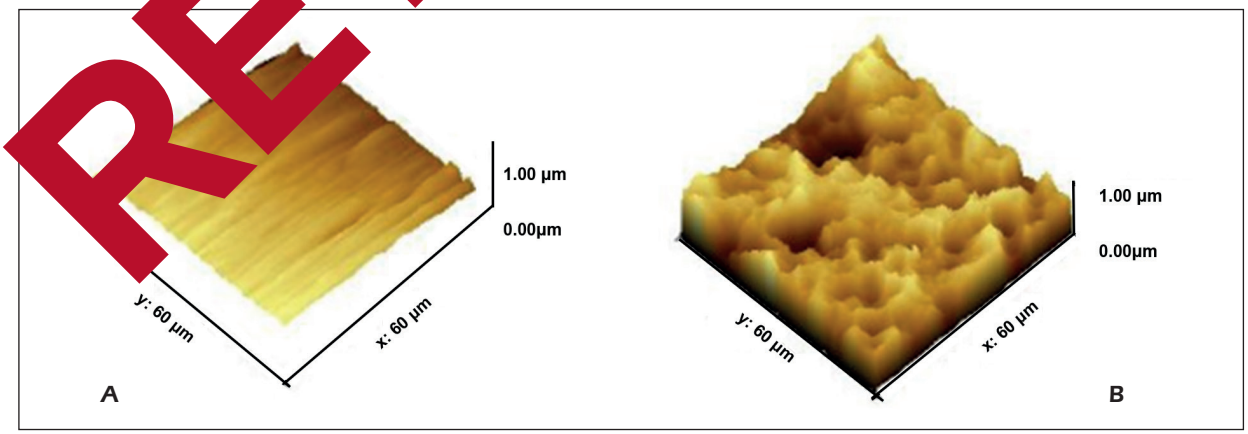

Figure 2. AFM observation of the three-dimensional structure of surface topography in both groups. **A**, PT group, **B**, NT group. **A**, Three-dimensional structure of surface topography of smooth PT group: visible scratch caused by mechanical polishing. **B**, Three-dimensional structure of surface topography of NT group: visible convex-concave nanometric structure.

Table II. Average roughness and contact angle of samples in both groups.

Sample	Average roughness (Rz/nm)	Contact angle (°)
PT	183.4±13.8	46.3±5.4
NT	424.6±20.4	15.8±4.2

Note: $p<0.05$ in comparison between the PT group and NT group.

Analysis of Detection Results of Icariin Loading Amount and Rule of Early Release

The curves of cumulative release of icariin in the first 14 d and 4 h in the PT-ICA group and NT-ICA group are shown in Figure 3. The curve of cumulative drug release in the NT-ICA group was higher than that in the PT-ICA group (Figure 3A). The cumulative drug release amount in the NT-ICA group (14.68 μg) in the first 14 d was about twice that in the PT-ICA group (6.85 μg). In addition, the trends of drug release curves in both groups were similar. In the first 4 h (quick-release phase), the release amounts in both groups were significantly larger, and it was sustai higher in the NT-ICA group in the first 4 h, w it was relatively unstable in the PT-ICA gr (Figure 3B). In the slow-release pha he relea amounts in both groups were lin ll, and more stable in the NT-ICA gr than t in the PT-ICA group (Figure 3A).

The curves of cumulative release percentage of icariin in the first 14 d and 4 h in the PT-ICA group and the NT-ICA group are shown in Figure 4. The curve of cumulative release percentage in the NT-ICA group was flatter than that in the PT-ICA group (Figure 4A). This observation indicated that the drug release was slow and there was a sustained drug-release effect. The above characteristics became more evident in th rapid-release phase (Figure 4B). In the N the cumulative release percentage he first 4 as lower, and the release was slo In the slo e-lease phase, the sustaine ug r e in th T-ICA group lasted until , which w than that in the PT-ICA up (5 d igure).

Discu

Preparation o iin/NT Composite C

he best sign of the success of dental implan- on is osse egration at the interface between b tissues implants[18]. However, the bioactiv m implants is low, and the osseointegration lasts for a long time after implantation. In particular, some metabolic diseases, such as betes mellitus and osteoporosis, can lead to abnormal bone structure and metabolic disorders, and interfere in the early osseointegration of implants[19,20]. Therefore, promoting osteogenesis around implants is a key issue after implantation.

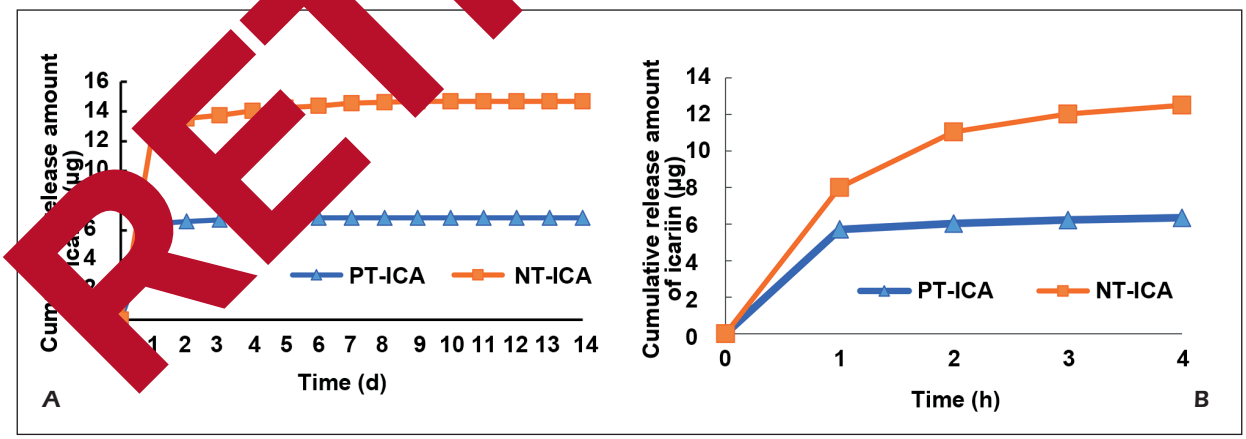

Figure 3. Curves of cumulative release amount of icariin in PT-ICA group and NT-ICA group. **A**, Curves of cumulative release amount in the first 14 d in PT-ICA group and NT-ICA group. **B**, Curves of cumulative release amount in the first 4 h in the PT-ICA group and NT-ICA group. **A**, Curves of cumulative release amount of icariin in the first 14 d in PT-ICA group and NT-ICA group. The curves of cumulative release amount in both groups can be divided into quick-release phase and slow-release phase, which is higher in the NT-ICA group than that in the PT-ICA group. **B**, Curves of cumulative release amount of icariin in the PT-ICA group and NT-ICA group in the quick-release phase (the first 4 h). The release amount is sustainably higher in the NT-ICA group in the first 4 h, while it is relatively unstable in the PT-ICA group.

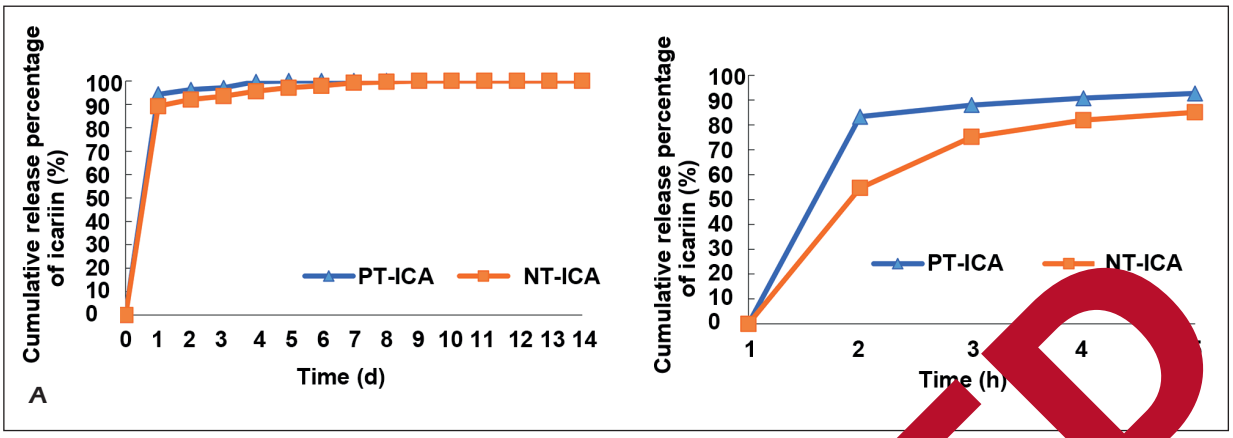

Figure 4. Curves of cumulative release percentage of icariin in PT-ICA group and NT-I group. Curves of mulative release percentage in the first 14 d in PT-ICA group and NT-ICA group. **B**, Curves of cum e r e percentage in the first 4 h in PT-ICA group and NT-ICA group. **A**, Curves of cumulative release percenta ica e first in the PT-ICA group and NT-ICA group. The curve of cumulative release percentage in the NT group is r th at in the PT-ICA group, and the cumulative drug release lasts for 9 d in the NT-ICA group and T-ICA gro urves of cumulative release percentage of icariin in the PT-ICA group and NT-ICA group in the q k-re phase (the t 4 h). The curve in the NT-ICA group is slower and more sustained than that in the PT-ICA group.

NT is an excellent local drug carrier characterized by good mechanical and chemical properties and biocompatibility[21-24]. It can promote the adhesion, proliferation and osteogenic d entiation of osteoblasts and bone marrow s n cells[25-28]. It can also absorb and contain me cations, so it is an excellent local g carrie Previous studies[29,30] mainly foc e for mation of the composite coati hroug oading antibacterial agents or acti po the surface of the nanot , but are a few reports on loading os nic drug the surface of nanotube[33].

Icariin is a l expensi ug with a stable nature, which stimulate th ivity of osteogenic trans ning f or in bone marrow stem cells and i the transformation of stem cells into o bla . Ica effectively enhance the p on o nic functional markers, p ote t secreti of osteocalcin, increase the a ity phosphatase, and induce the syn of type I collagen and osteoblast-specific tra ption factor (Osterix), thereby promoting the d erentiation and mineralization of osteoblasts[36,37]. In this work, icariin was loaded onto NT to prepare the icariin/NT composite coating, which synergized the osteogenic effect of drug and NT, thus further accelerating the early osseointegration process of implants.

The results obtained from previous studies showed that NT with a diameter of 80 nm presented strong hydrophilicity with a better effect promoting e adhesion of osteoblasts and b mesenc nal stem cells, and stronger osteog [38,39]. In this work, therefore, anodic oxidation parameters were explored and sted via preliminary experiment, and NT wi diameter of 80 nm was prepared. We used soaking method[40] which is characterized by simple and effective operation and a low risk of sample contamination compared with chemical coupling method[41] or dropping method[42]. It was found that the drug loading amount was larger with uniform distribution, and the drug release lasted for a longer time in NT drug-loaded composite coating formed after soaking in high-concentration soaking solution (2×10^{-3} mol/mL) than those in the coating formed after soaking in low-concentration soaking solution (1×10^{-3} mol/mL). Therefore, the drug was loaded using the icariin soaking solution in a concentration of 2×10^{-3} mol/mL. Moreover, the results of AFM and contact angle detection revealed that NT had greater roughness and stronger hydrophilicity, so NT can make the drug solution spread more uniformly on the surface of the sample, which has a high drug loading rate.

Drug Loading Amount and Rule of Early Release of Icariin/NT Composite Coating

Icariin possesses excellent properties of osteoinduction and osteogenesis. Its bioavailability is low and half-life period is as short as about 1-2 h[43]. Therefore, it is needed to increase the load-

ing amount of icariin and prolong the release time of icariin, thus improving the bioavailability and better exerting the osteogenic effect.

According to previous studies, the drug-loaded coating of implants has a larger drug loading amount than the smooth PT surface. It was shown that these can sustainably release the drug after implantation, and continuously provide a sufficient amount of medication for the surrounding tissues[44]. In this work, the amount of early drug release of icariin/NT composite coating was detected, the drug-loading amount was calculated, and the rule of early release was analyzed. The results showed that, compared with smooth PT surface, NT could provide higher drug release concentration and larger drug loading amount with a lower release rate and sustained-release effect. The above results are similar to those in previous studies on nanotube drug-loaded composite coating.

The drug loading mechanism of icariin/NT composite coating was analyzed, and it was found that the tubular structure of NT could provide larger surface area and volume to absorb more drugs on the surface and inside[45], thus increasing the drug loading amount effectively. The sustained-release effect attributes to the fact that the tubular structure of NT can limit the release of the drug[46], thus reducing the drug release rate and prolonging the release time. Therefore, the diameter and length of NT can be adjusted to obtain a larger drug loading amount and achieve sustained drug release, which provides sustainably high drug concentration for tissues around implants, thereby enhancing the early osseointegration.

The release of icariin from NT can be divided into two phases, namely early quick-release phase in the first and late slow-release phase in the following. In the quick-release phase, a large number of drugs loosely binding to the NT surface are quickly dissolved and released from the surface via physical absorption, which is the typical quick-release phase of a large number of drugs. In the slow-release phase, drugs loaded in the depth of NT are released via diffusion[47], in which a small number of drugs are sustainably and slowly released.

In the quick-release phase, there are two effects of icariin/NT composite coating on the osseointegration of implants. The quick release of a large amount of icariin in the coating can provide a sustainable and high drug release amount in the early stage (4 h), thus enabling icariin to exert an osteoinduction effect on bone mesenchymal stem cells, promoting the proliferation of bone mesenchymal stem cells and inducing the differentiation into osteoblasts[48,49]. Previous studies[50] demonstrated that icariin can reduce the number of autophagosomes and the level of mammalian microtubule-associated protein-1 light chain 3 (LC3-II). In the early stage (the first 3-6 h) of osteogenic differentiation of bone mesenchymal stem cells, the dramatic increase in the level of LC3-II and rapid degradation of autophagosomes can be seen[51], so bone mesenchymal stem cells can promote osteogenic differentiation of bone mesenchymal stem cells by reducing the number of autophagosomes and the level of LC3-II in the early stage. Therefore, a large amount of icariin is quickly released from the icariin/NT composite coating in the early stage, which can reduce the number of autophagosomes and the level of LC3-II, thus exerting an early osteoinduction effect. In addition, the early stage (the first 3-6 h) of osteogenic differentiation of bone mesenchymal stem cells is a key window phase in the whole process of osteogenic differentiation, and enhancing early osteogenic differentiation of bone mesenchymal stem cells can also effectively promote the late osteogenic differentiation[51,52]. On the other hand, the NT structure in the coating is able to promote the adhesion and spreading of bone mesenchymal stem cells and osteoblasts on the surface of implants in the early stage[28,53,54]. The early cell adhesion can form cytoskeleton and focal adhesion, and mediate transmembrane signal transduction, thus activating the expression of regulator gene and initiating the later cell growth and differentiation[55,56].

Recent studies[57] showed that bone mesenchymal stem cells in the bone regeneration region had diverse sources, which could be derived from its surrounding tissues (soft tissue and bone marrow) and systemic surrounding tissues. Therefore, it can be speculated that the implants with icariin/NT composite coating first directly contact stem cells in surrounding soft tissues and bone marrow after implantation into the jaw, and then bone marrow stem cells are further collected around the implantation region through the nanometric structure and induction effect of icariin. In summary, a large amount of icariin quickly released from the icariin/NT coating can exert an osteoinduction effect on bone mesenchymal stem cells in the quick-release phase, and the surface structure of nanotube can promote adhesion of bone mesenchymal stem cells and osteoblasts in the

early stage, both of which can synergistically enhance the early osteogenesis in the quick-release phase, and are expected to play an effective promoting role in the later osteogenic differentiation of bone mesenchymal stem cells. In the slow-release phase, the drug release rate of NT is lower, but the drug release amount is larger with a sustained-release effect than that of smooth PT surface, and the drug release can last until 9 d. Studies[37,58] have demonstrated that, when icariin acts on osteoblasts for 7 consecutive days, it displays definite effects of promoting differentiation and mineralization of osteoblasts. At the same time, NT can also promote proliferation and differentiation of osteoblasts[25,26]. Therefore, icariin and NT composite coating can still synergize in the later stage, to jointly promote the early osteogenesis in the slow-release stage to facilitate early osseointegration. In this work, the traditional Chinese medicine icariin with an effect of osteoinduction was loaded onto the surface of TiO_2 nanotube to form the composite coating, the amount of early release of the drug on the coating was detected, and the drug loading amount and rule of early drug release were evaluated. The results revealed that the drug loading amount of ic TiO_2 nanotube composite coating was relati large with a sustained drug-release effect, wh could provide a continuously high concentrati of drug in the early stage after im ement Moreover, icariin and TiO_2 na ube ca exert a synergistic osteogenic effe and slow-release phases herefo ariin/TiO_2 nanotube composite c g can im e the early osteoinduction a y mplant a promote the early osseoi gration een the implant and the bone t e, laying a fo tion for shortening the i tati ycle.

sions

The i NT composite coating can exert ergistic effect in the quick-release phase and s release phase through loading drugs with ost enic feature onto the surface of the nanotube, thus promoting early osteoinduction of implants. The coating can be prepared easily, and has larger drug loading amount and sustainably higher drug release amount with a sustained-release effect in the early phase than the method of directly loading icariin onto the smooth surface, thereby promoting early osseointegration, which has a good application prospect.

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Conflict of Interests

The authors declare that they e no c t of in st.

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