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Titania nanotube delivery fetal bovine serum for enhancing MC3T3-E1 activity and osteogenic gene expression

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ABSTRACT

Titania nanotube (TNT) delivery of fetal bovine serum (FBS) was conducted on titanium (Ti) to enhance bone tissue repair. Scanning electron microscopy (SEM) and energy dispersive spectrometer (EDS) showed FBS increased the tube wall thickness and decreased the tube diameter. Attenuated total reflectance Fourier transform infrared further confirmed that FBS completely covered the TNT and changed the surface composition. Water contact angle tests showed TNT/FBS possessed hydrophilic properties. Compared to original Ti, the TNT/FBS group had more attached osteoblasts after 2 h and enhanced filopodia growth at 0.5 h. Significantly, more osteoblasts were also observed on TNT/FBS after 7 d culturing. FBS was released steadily from TNT; about 70% of FBS had been released at 3 d and 90% at 5 d, as shown by the bicinchoninic acid method. TNT/FBS also enhanced subsequent osteoblast differentiation and gene expression; the quantum real-time polymerase chain reaction test showed that TNT/FBS up-regulated alkaline phosphatase and osteocalcin gene expression at 7 d and 14 d. Therefore, TNT/FBS delivered sustained in situ nutrition and enhanced osteoblast activity and osteogenic gene expression.

1. Introduction

Titanium (Ti) and its alloy are widely used in orthopedic implant applications due to their corrosion resistance and mechanical properties [1]. However, the weak conjunction at the bone-implant interface always leads to micromotions and cause further implant loosening [2]. In the US each year, approximately 600,000 fractures develop delayed union and 100,000 develop nonunion [3,4]. The bone-tissue repair process is a complex biochemical process, and sufficient nutrition plays a key factor in wound repair. Poor nutrition induced activation of the intrinsic coagulation system, causing thrombus formation [5]. On the contrary, the tooth-and-bone implants healed fastest when saliva and blood supply were adequate [6]. Therefore, new bone formed only where adequate nutrition existed [7,8].

In tissue engineering, fetal bovine serum (FBS), human serum and platelet lysate were always used for guaranteeing the nutrition supply [9]. All of them were ideal cell-growth supplement, containing high levels of nutrients and growth factors. In the clinical operation, bone grafts that were soaked in the patient's blood before implantation decreased the immune response. On the other hand, such treatment also supplied nutrition to bone cells and enhanced the wound repair. Researchers have found that FBS-pre-coated Ti had significantly higher cell adhesion [10,11], and FBS could further enhance cell proliferation and differentiation.

Much research has directly used growth factors to stimulate cell differentiation. However, directly delivering growth factor has a number of problems. Although the use of growth factors in animal experiments was valid, its use in tumor and diabetes patients may initiate excessive tumor switch and make the condition worse. So at present, growth factor application is limited to animal experiments and has not yet been applied in clinical situations. Actually, osteoblasts secrete growth factors themselves, and supplying enough nutrition could initiate a cascade of proliferation and differentiation signal pathways.

Therefore, implants that automatically provide nutrition to surrounding issues could enhance bone repair. Titania nanotube (TNT) is one proper structure to deliver the nutrition for its semi-closed tube structures [12]. Compared to the plain surface, the tube structure has larger surficial area and can hold more functional molecules to release. Various functional molecular could be sealed in TNT, such as functional proteins, growth factors, and anti-inflammatory drugs [13]. For example, Hu et al. [14] loaded bone morphogenetic protein 2 (BMP2) onto TNT to regulate the differentiation of mesenchymal stem cells. Aninwene et al. [15] loaded penicillin/streptomycin to reduce infection

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and dexamethasone for anti-inflammation, and Yao et al. [16] loaded penicillin-based antibiotics and achieved similar results. All the results showed the TNT structure could serve as an in situ sustained-release container.

Meanwhile, TNT could also enhance both osteoconductivity and osteoinductivity. The array of tubular structures on Ti alloy promotes bonding to bone, due to the high surface area and the ability for cell interlocking [17,18]. Furthermore, the geometry of the nanotube structure can regulate the bone mesenchymal stem cell osteogenic differentiation [19]. Popat et al. [20] placed TNT-modified implants in male Lewis rats for 4 weeks, and histological analysis showed that the TNT surface enhanced alkaline phosphatase activity and bone matrix deposition. Wilmowsky et al. [21] also found that TNT-modified implants influenced bone formation and bone development by enhancing osteoblast function in pigs. In addition, nanotube structure can be manufactured into any shape, and the tube diameter size can be accurately controlled at any value between 10 and 250 nm [22].

The aim of this study is to deliver the fetal bovine serum with TNT structures, we first created a TNT structure on Ti, and then FBS was lyophilized in TNT for in situ sustained release. We designed four experimental groups to contrast effects: 1) original Ti; 2) Ti modified by FBS (Ti/FBS); 3) TNT; and 4) TNT modified by FBS (TNT/FBS). Osteoblast MC3T3-E1 activity and osteogenic gene expression were measured for each experimental group.

2. Materials and methods

2.1. Specimen preparation

Pure Ti (Zhongtian Co., China) was cut into 2 mm × 10 mm × 10 mm pieces, and the plate surface was polished with No. 200 to No. 4000 silicon carbide sand paper. Highly ordered TNT were prepared by using a two-electrode cell with pure Ti sample as the working electrode and platinum foil as the counter electrode at 25 °C. The anodization process was performed at 40 V in a solution of 0.5 wt.% NH_4F and 2 ml H_2O in ethylene glycol for 2 h. The Ti/FBS and TNT/FBS samples were prepared by separately immersing Ti and TNT samples in FBS (Sigma, USA) at 4 °C for 24 h; these samples were then lyophilized.

2.2. Cell cultures

Mouse pre-osteoblast cell line MC3T3-E1 cells were cultured in alpha minimum essential media (Invitrogen, USA) supplemented with 10% FBS (Sigma, USA), 100 U/mL penicillin, and 100 mg/mL streptomycin. The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The cultured cells were detached by trypsinization (0.25% trypsin–EDTA), suspended in fresh culture media and used for the experiments described below. Osteoblasts were seeded at a density of 1×10^4 cells/well in a 24-well plate containing the samples.

2.3. Characterization

The morphology and element composition of the samples was monitored by scanning electron microscopy (SEM; HITACHI S-4800, Japan) with energy dispersive spectrometer (EDS). The surface functional group composition was investigated using attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR; Thermo Nicolet 6700, USA) at 4 cm^{-1} resolution. The dynamic contact angle analysis using double-distilled water was performed with a JC2000D drop-shape analysis system (Powereach Co., China). The initial distilled water volume was 5 μl, and the measurements were performed at ambient temperature.

2.4. Cell number counting and morphology

For the initial adherent cell number test, after culturing for 0.5, 1, and 2 h, the cells on the substrates were rinsed with PBS three times, detached from the substrates by trypsin, and counted using a hemocytometer (Hausser Scientific, USA). The proliferation of the osteoblasts was investigated by measuring the number of cells after 1, 3 and 7 d of culturing. The cells on the substrates were also rinsed with phosphate buffered saline (PBS) three times, detached from the substrates by trypsin and counted using a hemocytometer. For morphological observation, the osteoblasts cultured on samples were examined at 0.5 h by SEM. The cells that adhered to the surface were gently washed with PBS three times. Then, the substrates were fixed with 2.5% glutaraldehyde in PBS for 1 h at 4 °C. After being thoroughly washed with PBS, the cells were dehydrated through a series of graded ethanol and then critical-point dried. The surface was then gold sputter coated in a vacuum and examined.

2.5. FBS-releasing property

Since protein is the main component of FBS [23], the protein-releasing property reflected the FBS-releasing property. Protein determination was measured using the bicinchoninic acid (BCA) method with a BCA kit (Tiangen, China). Both Ti/FBS and TNT/FBS groups were immersed in PBS, measured at 1, 3, 5, 7, 9, 11, and 14 d for sustained release.

2.6. Gene expressions

After 7 and 14 d culture, the expression levels of osteogenesis-related genes were measured using quantum real-time polymerase chain reaction (qRT-PCR). The cells were harvested using TRIzol (Gibco, USA) to extract RNA. An equivalent amount of RNA from each sample was reverse transcribed into complementary DNA (cDNA) using the Superscript II first-strand cDNA synthesis kit (Invitrogen, USA). The qRT-PCR analysis of genes included runt-related transcription factor 2 (Runx2) (5′-GCCGTAGAGAGAGCAGGGAAGAC-3′and 5′-CTFFCTTGGATTAGGGAGTCAC-3′), alkaline phosphatase (ALP) (5′-TTTGCTACCTGCCTCACTTCCG-3′and 5′-GGCTGTGACTATGGGACCCAG-3′), type 1 collagen (Col-I) (5′-CGTGACCAAAAACCAAAAGTGC-3′and 5′-GGGGTGGAGAAAGGAACAGAAA-3′) andosteocalcin (OCN) (5′-AGACTCCGGCGCTACCTCAACAAT-3′and 5′-CAGCTGTGCCGTCCATACT-3′). Gene analysis was performed on the Applied Biosystems 7500 (USA) using the Real MasterMix (SYBR GREEN) kit (TIANGEN Biotech Inc., China). The expression levels of the target genes were normalized to that of the housekeeping gene GAPDH (5′-GGCACAGTCAAGGCTG AGAATG-3′and 5′-ATGGTGGTGAAGACGCCAGTA-3′).

3. Results

3.1. Characterizations of FBS-coated TNT

The SEM images of samples are shown in Fig. 1. Both Ti and Ti/FBS showed a relatively flat surface. For TNT morphology, the size of the tube was approximately 150 nm in diameter, 15 nm in wall thickness and 3.5 μm in length. The TNT/FBS had a smaller diameter (70 nm) and thicker wall thickness (50 nm). FBS modification did not change the tube length. EDS test was taken for TNT and TNT/FBS from the side view. Only Ti peaks were observed for TNT sample. While C, N and O peaks were clearly observable on TNT/FBS, these emerged peaks resulted from the FBS stored in TNT.

The ATR-FTIR spectra also differed among Ti, Ti/FBS, TNT, and TNT/FBS (Fig. 2). For the original Ti sample, the band at 740 cm^{-1} was attributed to Ti–O stretching vibrations. For the original TNT, the bands at 3409 and 1650 cm^{-1} were attributed to the hydroxyl group on the surface of TNT, which likely formed during the anodization