In situ delivery of nutrition using titania nanotube for promoting osteoblast proliferation and differentiation

Jing Peng¹, Xinming Zhang², Jin Cui², Xiang Liu²

¹Airport college, Civil Aviation University of China, Tianjin, 300300, China
²Tianjin Product Quality Inspection Technology Research Institute, Tianjin, 300384, China

Correspondence: Xinming Zhang
E-mail: xinmingmail@163.com
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Nutrition supply plays a key role in bone repair processes, but supplementing nutrition in situ is challenging with standard surgical implants. To enhance bone tissue repair, titania nanotube (TNT) containing fetal bovine serum (FBS) was used as a nano blood vessel to investigate sustained in situ nutrition supply. Scanning electron microscopy (SEM) showed TNT possessed nano-porous structure and FBS increased the tube wall thickness and decreased the tube diameter. Energy dispersive spectrometer (EDS) showed FBS had initial burst release and then sustained release for two weeks. The sustained release FBS enhanced osteoblast proliferation at 7 d by fluorescence observation. TNT/FBS also enhanced subsequent osteoblast differentiation; the alizarin red staining test showed that TNT/FBS enhanced osteoblast mineralization. In conclusion, TNT/FBS modification promotes osteoblast proliferation and differentiation.

Keywords: Titania nanotube; FBS sustain release; In situ nutrition supply; Osteoblast


Introduction

Titanium (Ti) and its alloys are commonly used in orthopedic fields, because they possess good biocompatibility and excellent mechanical properties. However, for hard tissue replacement, atrophic nonunion potentially causes surgical failure [1, 2]. Atrophic nonunion is caused by abnormal angiogenesis, which cause fibrous tissue formation [3]. Angiogenesis plays an important role in the process of bone repair: it promotes oxygen homeostasis, provides nutrients, and brings cells and biological mediators. Angiogenesis also affects intramembranous bone formation and endochondral ossification [4]. Therefore, inducing vascularization is necessary for implant survival, fusion, and future physiological function [5]. Research has shown that new bone was formed only where a vascular network existed [6-8].

Pre-vascularization is a method used on artificial bone scaffold to promote bone graft vascularization; this method has been shown to enhance bone growth [9, 10]. Although vessels play central roles in physiological processes, little is known of angiogenesis in bone repair during the initial process [11]. For titanium implants, current research involves using growth-factor modification on titanium to promote vascularization. For example, much research has focused on using growth factors that regulate the growth of endothelial cells to promote vascularization, since endothelial cells play a central role in angiogenesis, forming the capillary network and impacting blood circulation. Some growth factors have been used in bone-tissue engineering, such as vascular endothelial growth factor (VEGF) [12] and basic fibroblast growth factor (bFGF) [13, 14].

However, the current approaches to improve
vascularization are primarily from a biological perspective. Less research has focused on biomimetic vascular structure modification on titanium. If the implant has a vascular-like structure itself and also possesses the ability to spontaneously provide nutrition to surrounding issues, it may also enhance bone repair.

Titania nanotube (TNT) surface modification is a method to create an array of tubular structures; the TNT has the ability to promote cell interlocking for their high surface area. Furthermore, TNT can naturally act as a nano vessel, and the semi-closed tube can serve as a nano container for sustained release of nutrition. TNT surface modification has a number of additional benefits. For manufacturing, the nanotube structure can be made into any shape, and the tube diameter size can be accurately controlled at any value between 10-250 nm. For biocompatibility, the geometry of the nanotube structure can regulate the bone mesenchymal stem cell osteogenic differentiation. Therefore, TNT surface modification is a potential method for inducing angiogenesis.

As the main nutrition in blood, fetal bovine serum (FBS) is the ideal cell growth supplement, containing high level of nutrients and growth factors. When combined, FBS and TNT can act as a blood vessel to supply nutrition in situ. This approach could further enhance cell proliferation and differentiation, leading to improved bone repair.

In this study, we created a TNT modification on titanium, and FBS was lyophilized in TNT to mimic vascular tissue. Generally, the TNT/FBS groups acted as in situ nano blood vessels to deliver sustained nutrition. This approach could further enhance cell proliferation and differentiation, leading to improved bone repair.

Materials and methods

Pure Titanium (Zhongtian Co., China) was cut into 2 mm × 10 mm × 10 mm segment, then using different grade silicon carbide sand paper to polish its surface. Highly ordered TNT were prepared at 25 °C, by using a two-electrode cell with platinum foil and titanium sample as the counter electrode and working electrode, respectively. The anodization process was performed at 40 V in a solution of 0.5 wt% NH₄F and 2 ml H₂O in ethylene glycol for 2 h. The TNT/FBS samples were prepared by separately immersing TNT samples in FBS (Sigma, USA) at 4°C for 24 h; these samples were then lyophilized.

Mouse pre-osteoblast cell line MC3T3-E1 cells were cultured in alpha-MEM (Invitrogen, USA) contained 10% FBS (Sigma, USA), which also added 100 mg/mL streptomycin and 100 U/mL penicillin. The cells were incubated in 5% CO₂ and 95% air, which is a humidified atmosphere at 37°C. 0.25% trypsin–EDTA was used to detach the cultured cells. Osteoblasts density of 1×10⁴ cells/well were seeded in a 24-well plate, which containing the samples.

The morphology and sample element composition was
monitored by scanning electron microscopy (SEM, Hitachi, Japan) with energy dispersive spectrometer (EDS). Since element C is the main component of FBS, the element C line sweep distribution of TNT/FBS during the FBS delivery process was measured at 0, 1, 5, 7 and 14 d.

Fluorescence microscope was observed at 7 d to measure the proliferation of osteoblasts. The cytoskeleton was stained with Phallodin-FITC (Sigma, USA) and cell nuclei were stained with DAPI (Sigma, USA). Alizarin Red staining was observed at 14 d to evaluated the osteoblast differentiation property, washed the cells with PBS three times and then fixed 1 h in 75% ethanol. The cell cultures were stained with Alizarin Red (Sigma, USA) of 40 mM for several minutes at 25°C, then captured the color images.

Results and discussion

Fig. 2 shows the SEM images of TNT and TNT/FBS. Both of them possessed a uniformly porous surface, the size of the tube was approximately 150 nm. Osteoblasts present in different morphologies depending on the structural characteristics of their substrates [19]. As shown in Fig. 3, the cell fluorescence microscopy was nearly the same: cell spreading on the substrate normally. It is believed that when implant comes in contact with tissue, the biological environment is important. After implantation, the surface of the implant is covered with a layer of protein first; the amount and conformation of proteins determined the proceeding cell reaction [20]. Hence, the behavior of serum protein adhesion on implant was directly influencing the osteoblast attachment.

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The nutrition release behavior of TNT/FBS is shown in Fig. 4, which indicated a sustained release during the 7 d period, which contains a 5 d initial burst release interval. Peng et al [21] found that TNT/FBS showed a relatively sustained release behavior, reaching 90% at 5 d. And after 11 d, TNT/FBS released completely. The total protein deposited on TNT/FBS was about 30 ug/cm².

Sufficient nutrition supply also contributed to normal osteoblast differentiation. Some studies revealed that TNT/FBS up-regulated alkaline phosphatase and osteocalcin gene expression at 7 d and 14 d [21]. In this study, both TNT and TNT/FBS enhanced osteoblast differentiation; this was especially true for TNT/FBS, which possessed deeper colored staining result (Fig. 6).

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 the 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. In fact, osteoblasts secrete growth factors and could supply enough nutrition to initiate a cascade of differentiation signal pathways.

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**Conflict of interest**

The authors declare that there is no conflict of interest.

**References**