Cellular Compatibility of a New Tantalum-Niobium Scaffold Material

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Abstract

[Objective] To determine the cellular compatibility of porous tantalum-niobium (Ta-Nb) material.

[Method] Rabbit osteoblasts were co-cultured with porous Ta-Nb material. The cell proliferation was detected by CCK-8 method, and the cell adhesion was observed under scanning electron microscope (SEM). The expressions of type-I collagen and osteocalcin were detected by RT-PCR assay.

[Results] CCK-8 detection indicated that the cell proliferation on the porous Ta-Nb material showed no difference from that of the control group (P>0.05). SEM revealed that a large amount of cells adhered onto the surface and in the pores of the material. The number of cells on the material surface increased obviously over time. RT-PCR assay showed that with the prolonging of the time of co-culture, the expression of type-I collagen was enhanced (P<0.05), while the osteocalcin expression exhibited no significant difference (P>0.05

[Conclusion] Porous Ta-Nb scaffold material can be used to promote the adhesion, growth and differentiation of osteoblasts with satisfactory cellular compatibility.

Introduction

Medical metal materials made of titanium (Ti), cobalt (Co), tantalum (Ta) and niobium (Nb) have found wide applications in orthopedic field and have achieved marked clinical effect. Great progress has been made in the clinical application of porous cobalt-based materials recently [1]. The new porous tantalum-niobium (Ta-Nb) scaffold material used in this article was developed by the Powder Metallurgy Research Institute of Central South University [2]. In the present work, the in vitro co-culture of rabbit osteoblasts and porous Ta-Nb scaffold material was performed to study the cellular biological behaviors such adhesion on the material surface, proliferation and expression. The results provide an experimental basis for medical application.

1. Materials And Methods

1.1 Main experimental materials, reagents and instruments: DMEM/F_{12} medium, fetal calf serum, 500U/ml penicillin, 500μg/ml streptomycin (Gibco Life Technologies, USA), CCK-8 cell proliferation and toxicity assay kit (KeyGEN BioTECH, Nanjing, China). TRIzol@Reagent (Invitrogen, USA). Porous Ta-Nb material (Powder Metallurgy Research Institute of Central South University) was disc-shaped, with the diameter of 11 mm and the thickness of 4 mm (see Figure 1). This material was featured by a 3-D porous structure, with the porosity of 60% - 65% [2]. The uniform porosity and the interconnections between pores were confirmed under SEM (MIRA3 TESCAN, Research Institute of Tsinghua University in Shenzhen). The surface of the material was rough and uneven after local magnification (see Figure 2). The porous Ta-Nb material was soaked and rinsed with distilled water, sterilized with autoclave and dried prior to use.

1.2 Separation and culture of rabbit osteoblasts (OB): Three fetal rabbits younger than 10-day old (Animal Experimental Center of Guangzhou Medical University) were killed by intravenous injection of excess anaesthetics (3% pentobarbital sodium) from the edge of the ear. After cleaning, the rabbits were soaked in 75% ethanol for 5 min for sterilization. The sterilized rabbit was placed on the clean bench, and the residual ethanol was removed. Under aseptic condition, the skin was sliced and the skull (parietal bone and frontal bone) was harvested with scissors. The skull was rinsed with PBS containing 500 U/ml penicillin and 500 μg/ml streptomycin for several times. The adhesive connective tissues and blood vessels were removed with another set of scissors and pincers and then sliced into small pieces of about 1 m^m3. These small pieces were digested with 0.25% trypsin at 37°C for 20 min, and the digestate was discarded to eliminate fibroblasts. Then the digestion solution containing 0.2% collagenase type I and 0.1% hyaluronidase was added for digestion for 5 cycles on a shaker at 37°C for 20 min. The cell suspensions at the 3rd, 4th and 5th cycles were centrifuged at 1000 rbp for 10 min, with the supernatant discarded. The precipitated cells were rinsed with serum-free F-12 medium, centrifuged and resuspended in F-12 medium containing 10% fetal calf serum. After gentle blowing, the cells were inoculated into the culture bottle with the density of 1×10^5/ml and then cultured in an incubator containing 5% CO_2 at 37°C. The culture medium was refreshed every 3d. The cells were digested with trypsin when the cells grew to 70% - 80% confluence, and cell passage was performed with the ratio of 1:2-1:3. The third-generation osteoblasts were co-cultured with the porous Ta-Nb material.

1.3 Co-culture of osteoblasts and porous Ta-Nb material Osteoblasts were inoculated to the porous Ta-Nb material placed in the F-1_2 medium containing 10% fetal calf serum under aseptic condition. The experiment included two groups: experimental group (Group A), with the co-culture of osteoblasts and porous Ta-Nb material, and control group (Group B), with single culture of osteoblasts. The culture was performed using 24-well plates in an incubator containing 5% CO_2 at 37°C, with the inoculum density of 5×10^5/well. The medium was refreshed every 3d.

1.4 Detection indicators

1.4.1 Detection of cell proliferation by CCK-8 assay Five replicates were set up for group A and B, respectively. Each well was added with 50μL of CCK-8 solution on day 1, 3, 5, 7 and 9. After incubation at 37°C for 4h, the liquid in each well was transferred into 96-well plate, with equal liquid volume in each well. The absorbance (A) was detected by ELIASA at 490 nm and the cell proliferation curve was plotted.

1.4.2 SEM observation The porous Ta-Nb material was taken out after being co-cultured for 1, 2 and 3 weeks, respectively. The porous Ta-Nb material was rinsed with PBS for 2 times, fixed with 2.5% glutaraldehyde for 2h, and dehydrated with graded series of ethanol. Then it was dried at the critical point in liquid CO_2. After metal spraying, the cell adhesion was observed under SEM.

1.4.3 RT-PCR assay The assay was conducted by referring to the method of Saqomonyants et al. [3]. After being co-cultured for 1, 2 and 3 weeks, 1×10^6 osteoblasts of group A were collected, respectively. When the primary rabbit osteoblasts (P0) grew to 90% confluence, the total RNA was extracted as blank control.

The cells collected at different time points were treated with 1 mL of Trizol reagent (Invitrogen) for total RNA extraction. The RNA was quantified with ultraviolet spectrophotometer (WPA UV1101) at A260/280>1.8. Two-step RT-PCR method was employed. The first-strand cDNA synthesis was performed according to the kit instruction (Fermentas). 25μL reaction system: 10x Taq buffer 2.5 μL, 25 mmol/L $MgCl_2$ 3.0 μL, 10 mmol/L dNTP 2.0 μL, Taq DNA Polymerase (1 U/μL) 1.0 μL, ddH2O 18.5 μL, template cDNA 1.0μL; forward primer and reverse primer (20 μmol/L), 1.0 μL each. PCR amplification program: 95°C predegeneration for 3 min, 1 cycle; annealing for 45 s (the annealing temperature was as follows), 72°C extension for 1 min, 30 cycles; final extension at 72°C for 7 min. Type-I collagen (Col-I): upstream primer 5'-CCCAACCAAGGATGCACTAT-3', downstream primer 5'-TGTTCTGAGAGGCGTGATTG-3', annealing temperature 52°C, amplified fragment 259 bp. Osteocalcin (OCN): upstream primer 5'-CATGAGAGCCCTCACA-3', downstream primer 5'-AGAGCGACACCCTAGAC-3', annealing temperature 59°C, amplified fragment 310 bp. Reference primer GAPDH: upstream primer 5'-ACGGATTTGGTCGTATTGGG-3', downstream primer 5'-CGCTCCTGGAAGATGGTGAT-3', annealing temperature 56°C, amplified fragment 239 bp. 4 μL of type-I collagen, osteocalcin and 8 μL of GAPDH amplification product were stained with ethidium bromide and treated with 2% agar gel electrophoresis, respectively. The gray value of different bands was determined with Quantity One, and semi-quantitative comparison was performed.

1.5 Statistical analysis Measurement data were expressed as x±s. One-way ANOVA was performed on the data of each group using SPSS 13.0, and SNK-q test was performed for intergroup comparison.

2. Results

2.1 CCK-8 The proliferation of osteoblasts on the surface of the porous Ta-Nb material in shown in Figure 3.

No significant difference in cell proliferation was found between the experimental group and the control group (P>0.05).

2.2 SEM observation The SEM photographs of osteoblasts co-cultured with the porous Ta-Nb material are shown in Figure 4. The morphological analysis showed that the osteoblasts were evenly distributed on the surface of the porous Ta-Nb material, in a fusiform shape or scale-like shape. The pseudopodia were fully extended and connected into sheets. With the prolonging of the time of co-culture, the osteoblasts on the surface of the material were increased and fused gradually.

2.3 Expression of type-I collagen and osteocalcin detected by RT-PCR After co-culture for 1, 2 and 3 weeks, the expression of type-I collagen was increased with statistically significant difference (P<0.05), while the osteocalcin expression had no significant difference (P>0.05). Specifically, the expression of type-I collagen after 1 week showed no significant difference from that of P0 (P>0.05), and the expressions after 2 and 3 weeks were both higher compared with P0 (P<0.05). The expressions of osteocalcin after 1, 2 and 3 weeks remained basically the same as those of P0. See Figure 5.

3. Discussion

3.1 Significance of 3D interconnected porous structure

Although metal materials have been widely applied in orthopedic surgery and relevant fields in recent years, the effective integration of metal implant into osseous tissue is still a challenge. Most of the present studies aiming to improve the biological stability and compatibility of graft materials concentrate on the physicochemical properties or surface morphology (porosity). It was indicated by experiments that rough surface is conducive to the capture of cellulose. The implant with rough and porous surface makes cell adhesion easier. The concentration of cellulose can induce the aggregation of osteoblasts. This can benefit the ingrowth of bone tissues into the implant, accelerate early-stage osseointegration and enhance the binding of the bone to the implant [4]. Thus the long-term stability of implant can be achieved by the "binding" of the bone to the implant [5]. Previous study indicated that when the prostheses with porous and thread surface were implanted into the bone of a host which is subjected to load, the prosthesis with porous surface had a larger contact area with the surrounding bone and the ingrowth of osteocytes into such prosthesis was faster [6]. Moreover, it was reported that the 3D porous surface can promote the proliferation of osteoblasts and improve the osteogenic capacity [7, 8, 9].

3.2 Influence of pore size and porosity on the ingrowth of osteocytes

The pore size and porosity of the porous material have a great influence on the ingrowth of osteocytes into the implant. The diameter of a mature osteocyte is about 20 μm, which means that if the pore size of the implant material is smaller than this, the ingrowth of osteocytes is not possible. The size of an osteon (bone matrix around the nucleus of an osteocyte) is about 60 μm - 80 μm, so the pore size should be at least larger than 80 μm. Some studies indicated that the interconnected porous structure with the pore size of 430μm - 650μm was favorable for the ingrowth of the osteocytes [10]. Porosity refers to the number of pores in unit volume, and the porosity favorable to the ingrowth of osteocytes is 75% - 85%. The 3D interconnected porous Ta-Nb material developed in this article had the pore size of 500 μm and the porosity of 60%-65%, which met the requirement for the ingrowth of osteocytes. But the pore blocking rate was higher, which hindered the ingrowth of osteocytes.

3.3 Mechanical property of the porous Ta-Nb material

Mechanical property is an important indicator used to evaluate the orthopaedic biomaterials. An ideal orthopaedic biomaterial cannot only facilitate the ingrowth of osteocytes, but also bear a certain load. Modulus of elasticity is a major indicator of mechanical property. The modulus of elasticity of the 3D interconnected porous Ta-Nb material was (815±5) MPa, which was close to that of human cancellous bone (0.1-1.5 GPa) [2]. Due to similar modulus of elasticity, the stress-shielding effect was smaller after implanting, thus ensuring a normal biological stress conduction.

After local magnification under SEM, some fractures were found in the porous Ta-Nb samples (Figure 2c), which influenced the mechanical property to some extent. Therefore, the porous Ta-Nb sample used in

this article still needs to be improved technically.

3.4 Comparison with existing studies

Generally, the golden standard for the comparison of cellular compatibility of different scaffold materials is tissue culture on plastic plate. [11] In this present work, the osteoblasts were co-cultured with the porous Ta-Nb material, and the cell proliferation on the porous material had no significant difference from the golden standard. In the study by Sagomonyants et al [3], the number of cells on the porous tantalum scaffold after 48-h culture was 12-16 times that of the golden standard, which was inconsistent with our result. This may be caused by the difference of porosity of scaffold materials. In this article, the Col-I expression detected by RT-PCR in the third week was higher than that of the golden standard, but agreed with that in Sagomonyants's study [3]. But in the study by Welldon et al [11], the Col-I expression detected by real-time PCR was decreased in the second week. The OCN expression showed no significant difference from that of the golden standard and agreed with that of Sagomonyants et al [3] and Welldon et al [8]. Through the semi-quantitative PCR assay adopted in this article, the data obtained showed a large variation. This means the significant difference of mRNA expression could be hardly determined even if there was any. Moreover, the mRNA expressions of bone matrix proteins might vary between different individuals. [4] The physicochemical property of the scaffold material may be another reason for the difference from the previous studies. The material used in this article was porous Ta-Nb, while that in the previous two studies was porous tantalum.

Conclusion

The porous Ta-Nb material used in this article is a scaffold material with good cellular compatibility, showing no adverse effect on the adhesion, growth and differentiation of osteoblasts.

Abbreviations

Co: cobalt; Col-I: Type-I collagen; Nb: niobium; OB: osteoblast; OCN: osteocalcin; Ta: tantalum; Ta-Nb: tantalum-niobium; Ti: titanium;

Declarations

Ethics approval and consent to participate

All the animal related experiments were approved by the Institutional Animal Ethics Committee of the Shenzhen Second People's Hospital, Shenzhen, Guangdong Province, China.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

JO performed the experiments, and performed data collection and analyses. ZD designed the study and participated in data analyses and manuscript preparation. KC performed data analyses. JX performed data collection. YL performed data collection. DW performed data collection. WL performed data collection. YZ contributed to study design. WZ contributed to study design.

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Not applicable.

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Figures

Figure 1

Porous Ta-Nb material

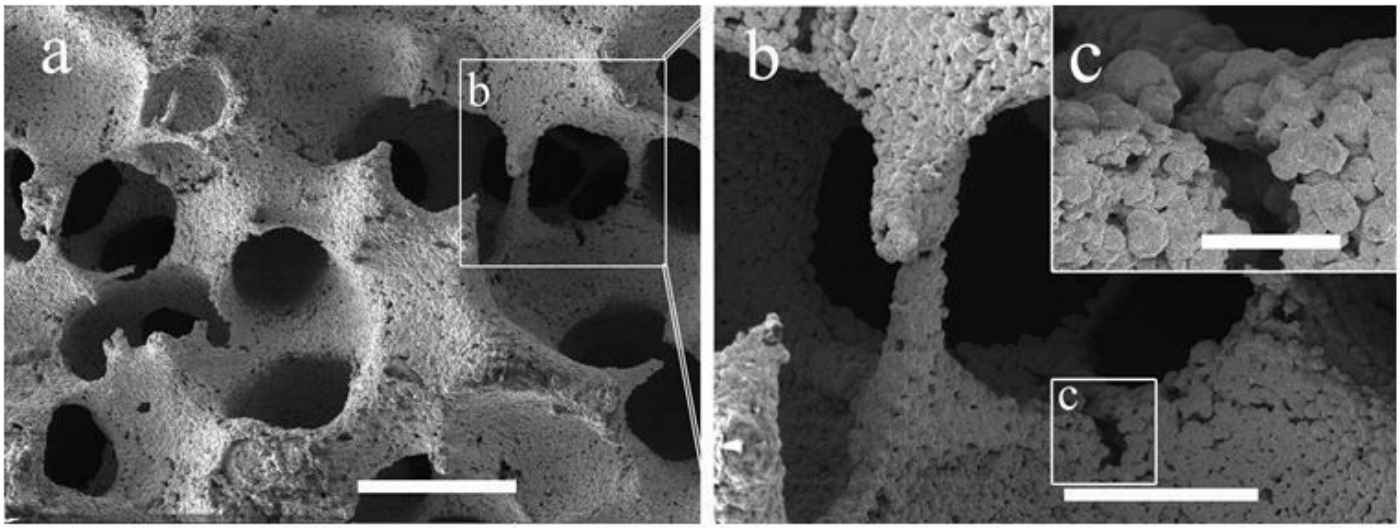

Figure 2

SEM photograph of porous Ta-Nb material a. Uniform porosity, with the scale of 500 μm; b. Interconnection between pores, with the scale of 200 μm; c. With the scale of 50 μm.

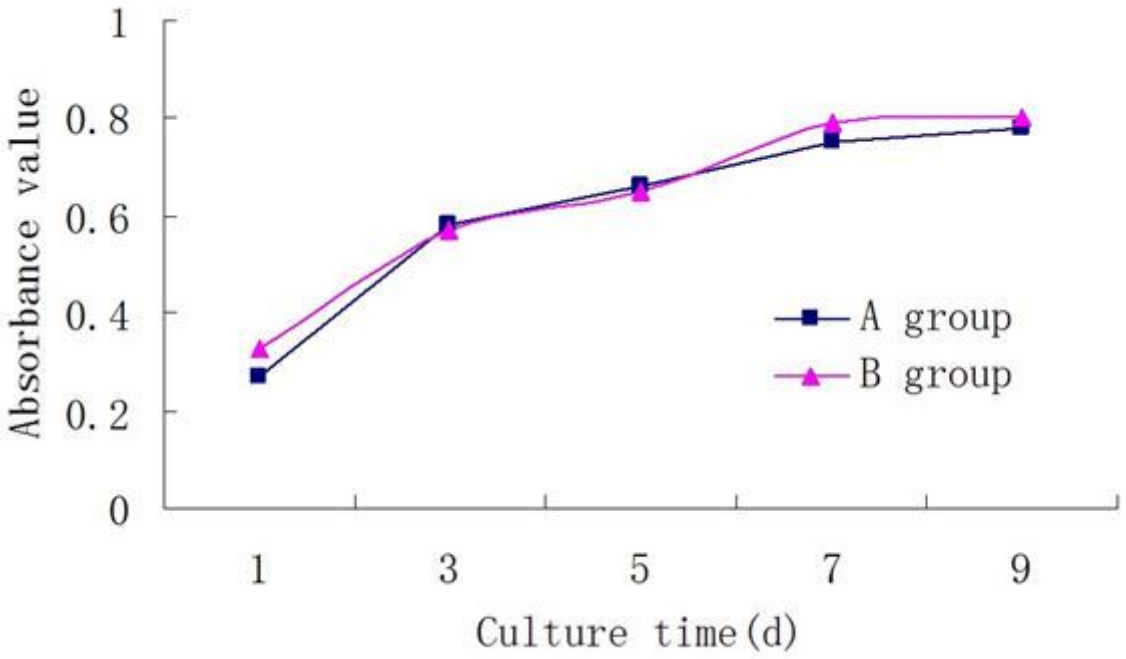

Figure 3

The proliferation of osteoblasts on the surface of scaffold material (group A) showed no significant difference from that on the plastic plate (group B) ($P>0.05$).

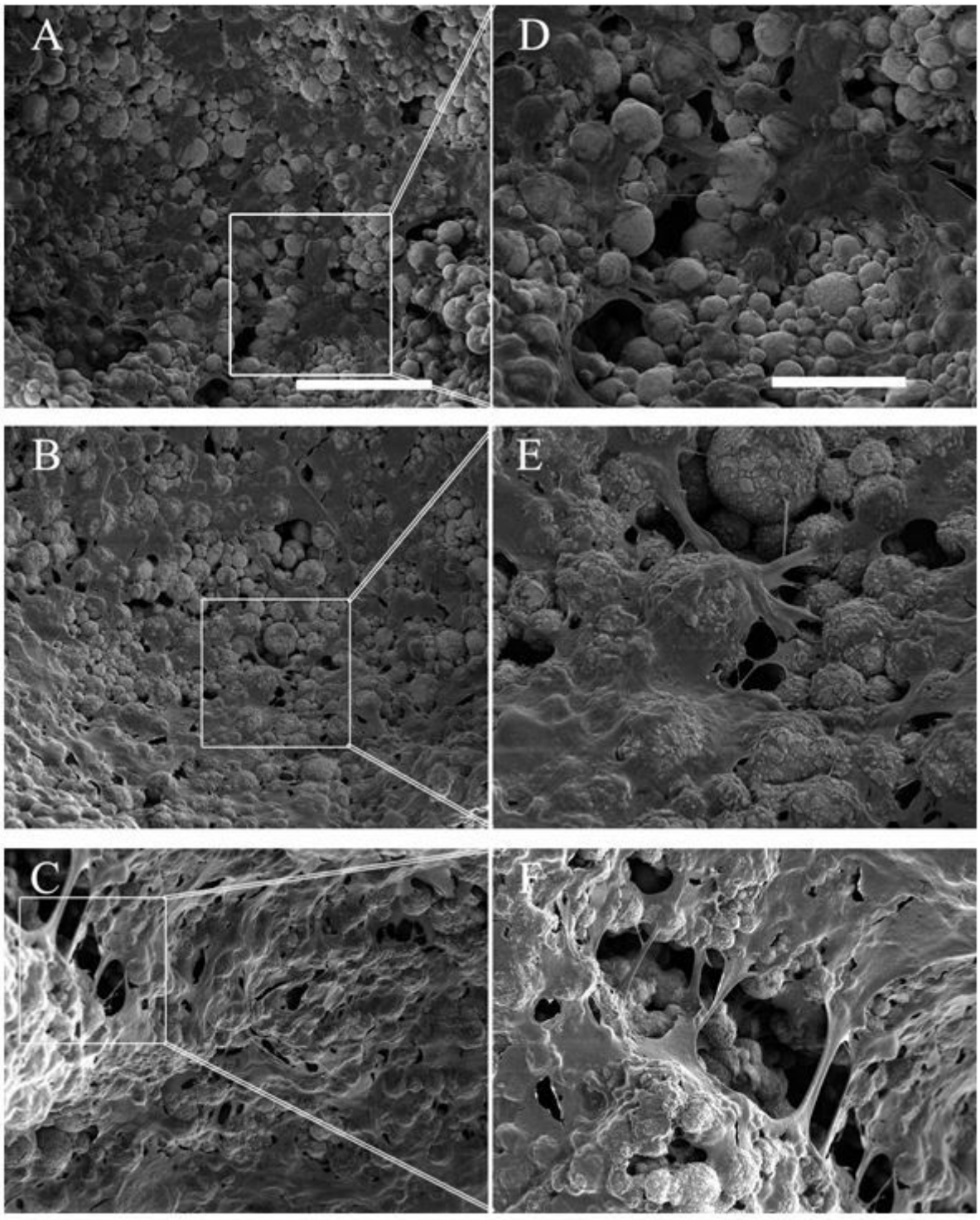

Figure 4

A, B and C are the SEM photographs of rabbit osteoblasts co-cultured with porous Ta-Nb material after 1, 2 and 3 weeks, respectively, with the scale of 100 μm; D, E and F are the magnified images of A, B and C, respectively, with the scale of 50 μm. With the prolonging of the time of co-culture, the number of cells adhering on the scaffold material increased remarkably.

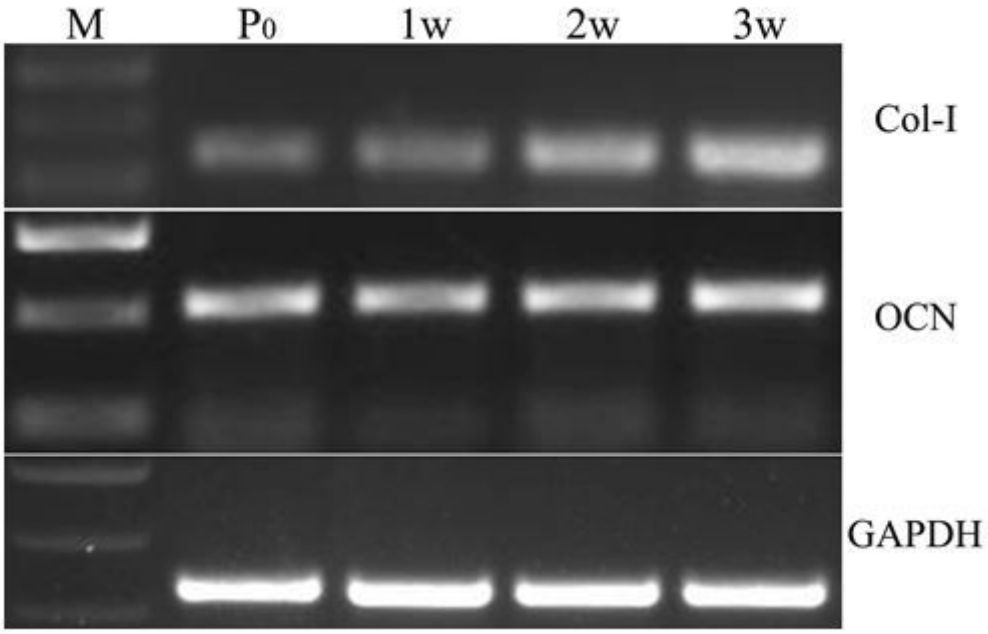

Figure 5

After co-culture for 1, 2 and 3 weeks, the expression of type-I collagen (Col-I) increased with statistically significant difference ($P<0.05$), while the osteocalcin (OCN) expression had no significant difference ($P>0.05$). The expression of type-I collagen showed no significant difference from that of P0 in the 1st week ($P>0.05$), but it was higher than the latter in the 2nd and 3rd week ($P<0.05$). The osteocalcin expressions were basically consistent with that of P0 in the 1st, 2nd and 3rd week, respectively.